410 Rec'd PCT/PTO 21 SEP 1999

FORM PTO-1390 (REV 1-98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: MSKP031USNP

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/381556

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/05505	20 MARCH 1998	21 MARCH 1997

TITLE OF INVENTION
RAPID PRODUCTION OF AUTOLOGOUS TUMOR VACCINES BY USING HSV AMPLICON VECTORS

APPLICANT(S) FOR DO/EO/US
FONG ET AL.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 US C 371
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 US.C 371
3. ☒ This express request to begin national examination procedures (35 US C 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U S C371(b) and PCT Articles 22 and 39(1)
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U S C 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U S C 371(c)(2))
7. ☒ Amendments to the claims of the International Aplication under PCT Article 19 (35 U S C 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
   b. ☐ have been transmitted by the International Bureau
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U S C 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U S.C 371(c)(4))
10. ☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C 371(c)(5))

Items 11. to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1 97 and 1.98
12. ☐ An assignment document for recording A separate cover sheet in compliance with 37 CFR 3 28 and 3 31 is included
13. ☒ A FIRST preliminary amendment
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification
15. ☐ A change of power of attorney and/or address letter
16. ☒ Other items or information:

Copy of the Abstract from the original application

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U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/381556	PCT/US98/05505	MSKP031USNP

17. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5))		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1070.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $930.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $790.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $720.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $98.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 670	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e))	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	40 - 20 =	20	x $22.00	$ 360	
Independent claims	4 - 3 =	1	x $82.00	$ 78	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 1108	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) +				$	
SUBTOTAL =				$ 1108	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f))				$	
TOTAL NATIONAL FEE =				$ 1108	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$ 1108	
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 1108 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees
A duplicate copy of this sheet is enclosed

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No 15-0610. A duplicate copy of this sheet is enclosed

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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Nancy J. Parsons
SIGNATURE

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NAME

40,364
REGISTRATION NUMBER

MSKP031USNP

BEFORE THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: FONG et al.

Serial No.: to be assigned (national phase of PCT/US98/05505)

Filed: herewith

For: Rapid Production of Autologous Tumor Vaccines by Using HSV Amplicon Vectors

PRELIMINARY AMENDMENT

Asst. Commissioner for Patents

Washington, D.C. 20231

Sir:

Preliminary to the examination of the above-referenced application, please make the following amendments:

In the specification:

On page 1, before line 1, insert

- -This application is a national phase of International Application Serial No. PCT/US98/05505 which claims priority from US Provisional Application Serial No. 60/044,005 filed March 21, 1997.- -

In the claims:

In claims 7, 12 and 14, line 1, delete "any of claims 1 to 6" and insert - -claim 1- -.

In claim 16, line 1, delete "any of claims 1 to 15" and insert - -claim 1- -.

In claim 17, line 1, delete "any of claims 1 to 16" and insert - -claim 1- -.

In claim 18, line 1, delete "any of claims" and insert - -claim- -.

In claim 22, line 1, delete "any of claims 1-21" and insert - -claim 1- -.

In claim 32, line 1, delete "any of claims claim 23-31" and insert - -claim 23- -.

In claims 33 and 34, line 1, delete "any of claims claim 23-32" and insert - -claim 23- -.

In claim 36, lines 1-2, delete "any of claims 1 to 22" and insert - -claim 1- -.

In claim 37, line 2, delete "any of claims 23 to 35" and insert - -claim 23- -.

REMARKS

The amendment to the specification is made to add a reference to the provisional application from which the PCT application of which this application is the national phase claims priority. The amendments to the claims correct multiple dependencies and typographical errors. No new matter has been added.

Applicants also enclose a replacement copy of the Abstract as filed with the original application, in case the Abstract was misplaced during processing.

Respectfully submitted,

Nancy J. Parsons
PTO Reg. No. 40,364
Patent Agent
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RAPID PRODUCTION OF AUTOLOGOUS TUMOR VACCINES BY USING HSV AMPLICON VECTORS

DESCRIPTION

The work described in this application was supported in part by NIH Grants Nos. CA76416, CA72632, HD 31300, DK53160, and PO1 CA59326. The United States government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Cytokine gene transfer to tumor cells has been used to increase local production of these immune modulating proteins, with the aim of enhancing tumor immunogenicity and consequent host recognition and elimination of tumor (Dranoff *et al.* 1993; Gansbacher *et al.* 1992). Production of irradiated, non-dividing tumor cells secreting cytokines such as Interleukin-2 (IL-2), gamma-interferon (γ-IFN), or granulocyte macrophage-colony stimulating factor (GM-CSF) represents a potential therapeutic strategy for treatment of malignant disease (Saito *et al.* 1994; Dranoff *et al.* 1993; Gansbacher *et al.* 1992), and one that is currently being evaluated in clinical trials (Lotze *et al.* 1994; Seigler *et al.* 1994; Rosenberg *et al.* 1992). Many methods have been examined for gene transfer (Davidson *et al.* 1993; Drazan *et al.* 1994; Yang *et al.* 1995; Paquereau & Le Cam, 1992; Jarnagin *et al.* 1992); the most successful have been those using retroviral vectors (Dranoff *et al.* 1993; Gansbacher *et al.* 1992).

An impediment to the production of autologous tumor vaccines has been logistic problems surrounding gene transfer to freshly harvested tumors. The most widely utilized approach for gene transfer to tumors relies on retroviral vectors, which are relatively inefficient and require replicating cells for gene expression (Wilson *et al.* 1988). The production of an autologous vaccine using retroviral vectors requires placing harvested tumor in tissue culture before *in vitro* transduction, selection, and isolation of the minority of cells in which gene transfer was successful. Such a process is therefore lengthy, expensive, and fraught with technical problems of establishing and maintaining primary cell culture. These difficulties have forced investigators to examine as alternative vaccine strategies the administration of established allogeneic cytokine-secreting tumor cell lines

(Patel *et al.* 1994), use of other vectors for gene transfer such as adenoviral vectors (Drazan *et al.* 1994; Yang *et al.* 1995), or the administration of cytokine-producing fibroblast cell lines along with the autologous tumor cells (Lotze *et al.* 1994).

Furthermore, it may be desirable to use multi-therapy for the treatment of many types of tumors, i.e, therapy in which cells are transduced to express more than one type of protein. To use retroviral vectors for such a process requires the construction of a specific vector for each combination of genes. Constructions of multi-gene vectors is complicated, which may place practical limits on the variations of multi-therapy which may become available.

It is an object of the present invention to provide a method for rapid and efficient production of autologous tumor vaccines expressing a plurality of immunomodulatory proteins.

It is a further object of the present invention to provide a method for rapid production of autologous tumor vaccines expressing chemokines, intercellular adhesion molecules or costimulatory factors, or combinations thereof, which can be completed within hours, for example in less than four hours, permitting rapid treatment of tumor patients.

It is still a further object of the invention to provide a method for rapid production with these autologous tumor vaccines which can be applied to tumor cells *in vivo* without requiring surgical removal of tumor material.

It is still a further object of the present invention to provide compositions useful in the methods of the invention.

SUMMARY OF THE INVENTION

In accordance with the present invention an autologous vaccine to tumor cells is produced by transducing the tumor cells with a herpes simplex virus amplicon containing the gene or genes for an immunomodulating proteins and an additional therapeutic gene to provide transient expression of the immunomodulating proteins by the cells. The tumor cells may be transduced with the herpes simplex amplicons *ex vivo* or *in vivo*. Exemplary immunomodulating proteins which may be used individually in the method of the invention include chemokines such as RANTES, intracellular adhesion molecules such as ICAM-1, and costimulatory factors such as B7.1. A particularly

important aspect of the present invention is the fact that tumor cells may be readily transduced with a combination of amplicons containing genes for two or more different immunomodulating proteins, for example interleukin-2 and interleukin 12 or RANTES and B7.1. This greatly facilitates the production of multiply-transduced cells for multi-targeted therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-E summarize the results of studies on the efficiency of gene transfer using HSV amplicons according to the invention;

Figs. 2A-C summarize the effects of irradiation on gene transfer efficiency;

Figs. 3A-C illustrate the tumoricidal activity splenocytes from mice treated by intrasplenic injection with HSV amplicon transduced tumor cells;

Fig. 4 summarizes the results of studies on the efficiency of gene transfer using HSV amplicons according to the invention;

Fig. 5 illustrates the effect of transduced cells on tumor growth;

Fig. 6 illustrates the effects of transduced cells on hepatectomy induced tumor formation;

Fig. 7 shows the amount of human ICAM-1 found in cell culture supernatants for transduced cells;

Fig. 8 shows the adhesion index for adhesion of lymphocytes to hepatoma cells transduced with HSVhICAM1 versus controls;

Fig. 9 shows tumor growth in rats injected with hepatoma cells transduced with HSV-hICAM1 versus controls;

Fig. 10 shows tumor nodules formed in rat liver after vaccination with radiated (nonviable) HSVhICAM1-transduced hepatoma cells followed by challenge with viable hepatoma cells;

Fig. 11 shows the structure of several HSV-immunomodulatory protein amplicons in accordance with the invention;

Figs. 12A-C show B7.1 expression in EL4 cells transduced with HSVB7.1 versus controls;

Figs. 13A and B show tumor size in intratumorally-treated tumors and contralateral tumors, respectively; and

Figs. 14A-D show CTL activity observed in splenocytes from mice receiving HSVB7.1 or HSVrantes alone or in combination, versus an HSVlac control.

DETAILED DESCRIPTION OF THE INVENTION

Herpes simplex virus (HSV) is a DNA virus capable of rapidly and efficiently infecting a wide variety of cell types (Leib & Olivo, 1993; Geller & Federoff, 1991). Plasmid-based viral vectors derived from HSV, termed amplicons, are easily constructed and packaged into viral particles. The present invention uses herpes simplex virus amplicons containing genes encoding for immunomodulating proteins to transduce tumor cells with high efficiency either *ex vivo* or *in vivo*.

As used herein, the term "immunomodulating proteins" refers to a class of protein or peptide molecules which, when expressed by a target cell, enhance the development of an immune response to that cell. The term includes cytokines, including chemokines; intercellular adhesion molecules, and costimulatory factors necessary for activation of B or T cells.

Cytokines which may be used as immunomodulating proteins in the invention include but are not limited to interleukins, such as interleukin-2 (IL-2), interleukin-12 (IL-12); interferons, for example gamma interferon (γ-IFN), granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α). The immunomodulating protein may also be a chemokine such as RANTES, which is a β or C-C chemokine, that functions as a chemoattractant and activator for monocytes and macrophages. Other C-C chemokines, such as MCP-1, -2, and -3, DC-CK1 and MIP-1α, -3α, -β and -3β, and α or C-X-C chemokines such as IL-8, SDF-1β, 8DF-1α, GRO, PF-4 and MIP-2 could also be used. Other chemokines useful in the method are C family, for example lympotactin and CX3C family, for example fractal kine, chemokines.

Intercellular adhesion molecules are transmembrane proteins within the Ig superfamily that act as mediators of adhesion of leukocytes to vascular endothelium and to one another. A preferred intercellular adhesion molecule for use in the invention is ICAM-1

(also known as CD54), although other cell adhesion molecules that binds to T or B cells, including ICAM-2 and -3 could also be used.

Costimulatory factors which may be used as the immunomodulatory protein in the present invention are cell surface molecules other than an antigen receptor and its ligand that are required for an efficient response of lymphocytes to an antigen. Examples of such costimulatory factors include B7 (also known as CD80).

HSV vectors systems are efficient vehicles for gene transfer to tumor cells. In experiments using HSVlac, over 50% of the target cells are transduced using an MOI of 1. The efficiency of transduction is further reflected by the high levels of IL-2 produced by HSVil2-transduced cells. Production of levels greater than 1 $\mu g/10^6$ cells/24 hour was found, which is more than 30 times that achieved by retrovirally-produced vaccines (Patel *et al.* 1994; Gansbacher *et al.* 1992). Additionally, the data from the experiments with HSVil2-transduced human tumors demonstrate that successful HSV-mediated gene transfer to freshly-isolated tumor cells can also be used to produce genetically-engineered cells that secrete significant amounts of bioactive IL-2.

A major advantage of using HSV vectors for gene transfer is the ability to transduce non-replicating or slowly replicating cells (Geller & Federoff, 1991). This physical property of HSV translates into important clinical advantages. Freshly isolated tumor cells may be transduced without the need to provide a tissue culture environment conducive to cell replication. This advantage is clearly demonstrated by the rapidity with which freshly harvested human tumors were transduced in the current experiments. Within 20 min, efficient gene transfer was produced, suggesting that vaccines prepared by this method could be ready for administration to patients within a single operative procedure. That HSV-mediated gene transfer is independent of cell division and is supported by a transduction efficiency that was not reduced by prior irradiation of tumor cells. Thus, gene transfer to tumor cells may be performed either before or after radiation according to irradiation source availability, providing greater flexibility in the clinical care of patients.

HSV-immunomodulatory protein amplicons and cells transduced with such amplicons are able to confer specific antitumor immunity that protects against tumor growth *in vivo*. The amplicons may be introduced indirectly by administration of transduced cells into a living organism or patient (mammalian, including human). Alternatively, the HSV-

immunomodulatory amplicon may be introduced directly into tumor tissue (e.g. by peritumoral injection) within a living organism or patient to generate an antitumor immunity which leads to reduction in tumor size. This latter approach is useful, for example, in the case of inoperable tumors.

In accordance with the present invention, HSV-immunomodulatory protein amplicons may be administered , directly or indirectly, as individual species in order to provide a therapeutic and/or prophylactic benefit. For example, as described in the examples set forth herein, it has been determined that administration of HSV-immunomodulatory protein amplicons encoding cytokines such as IL2, GM-CSF and RANTES, intercellular adhesion molecules such as ICAM-1 and costimulatory factors such as B7.1 all provide therapeutic benefit in the form of reduction or preexisting tumor size, a vaccine-effect protecting against tumor growth after a subsequent challenge, or both.

HSV-immunomodulatory protein amplicons may also be administered, directly or indirectly, with other species of HSV-immunomodulatory transduced cells or in combination with cytokine therapy. Such administrations may be concurrent or they may be done sequentially. Thus, in one embodiment of the invention, HSV amplicons or cells transformed with an HSV amplicon encoding an immunomodulatory protein are injected into a living organism or patient, after a pre-treatment with a therapeutically effective amount of a cytokine. Both HSVil2 and HSVgm-csf have been shown to have increased efficacy when administered following a pretreatment of γ-IFN.

In another embodiment of the invention, populations of HSV amplicons or cells transduced with HSV amplicons encoding a plurality of different immunomodulatory proteins may be coadministered to the subject. For example, populations of tumor cells transduced with HSVil2 and HSVil12 may be coadministered As shown in the examples, such coadministration is somewhat more effective than administration of individual populations. Coadministration of cells expressing these two cytokines appears to be most effective, however, when a single population of cells that has been transduced with two different cytokine-encoding amplicons is used.

Another example of the benefits of coadministration of a plurality of HSV-immunomodulatory protein amplicons is seen with the chemokine RANTES and the costimulatory factor B7.1, Although peritumoral administration of either HSVB7.1 or

HSVrantes resulted in tumor rejection is a significant number of test subjects, when HSV amplicons encoding these two immunomodulatory proteins are combined, an increased number of animals reject the tumors.

Populations of cells expressing two or more immunomodulatory proteins can be made either with separate amplicons species, one encoding each immunomodulatory protein, or which a single amplicon species encoding a plurality of immunomodulatory proteins. The ability to use separate amplicon species to transduce cells to produce multiple immunomodulatory proteins is a major advantage over prior methods, such as use of retroviral vectors, for introduction of genetic material into target cells. In these prior methods, the frequency of transduction is so low that no reasonable percentage of cells would be transduced with multiple genes if two or more separate viral vectors were used. Therefore, therapies of this type require the preparation of a unique and complicated construct containing multiple genes for each separate form of multi-targeted gene therapy. Using the method of the present invention, however, each target gene can be constructed in its own amplicon, and multi-transduced cells produced by simply mixing combinations of desired amplicon species.

In a broader sense, the invention provides a method for production of an autologous vaccine to tumor cells comprising transducing the tumor cells with one or more species herpes simplex virus amplicon containing the gene for an immunomodulatory protein and at least one additional therapeutic gene to provide transient expression of the immunomodulatory protein and the therapeutic gene product by the cells. As noted from the specific examples in this application, the additional gene may by a gene encoding a second immunomodulatory protein. However, the therapeutic gene product is not limited to immunomodulatory proteins, and may include any protein or peptide which it is desirable to have expressed by autologous tumor vaccine cells. Thus, for example, the gene might code for an enzyme which is used for pro-drug conversion (for example, thymidine kinase), or for a protein which promotes apoptosis (BAX or $BCLX_s$).

The invention also provides a method for inducing a protective immune response to tumor cells in a patient (animal or human) comprising the step of transducing the tumor cells with a herpes simplex virus amplicon containing the gene for at least one immunomodulatory protein to provide transient expression of the immunomodulatory

protein by the cells. The tumor cells may be transduced with the amplicon *ex vivo*, in which case the method further comprises the step of introducing the transduced tumor cells into the patient. The tumor cells may also be transduced *in vivo* by injecting the HSV amplicons into the site of the tumor cells.

The invention also provides HSV amplicons which contain the gene for one or more immunomodulatory proteins, and cells transduced with such amplicons.

The invention will now be further described with reference to the specific examples which follow. It should be understood, however, that these are merely offered as examples and are not intended to limit the scope of the invention. Thus, other immunomodulatory proteins not specifically mentioned, and other combinations of immunomodulatory proteins, including combination of three or more immunomodulatory proteins may be used and are considered to be with in the scope of the present invention as defined in the claims of this application.

EXAMPLE 1

Herpes viral vectors: *construction and packaging*: The replication defective HSV amplicon vector expressing human IL-2 was constructed by directionally cloning the gene, excised from r-IL-2 (Saito *et al.* 1994) with Sac I and Eco RI, into HSV PrPUC (Bergold *et al.* 1993) digested with the same enzymes. The HSV vector expressing ß-galactosidase (HSVlac) has been previously described (Geller & Breakefield, 1988). Both amplicon vectors were packaged as previously described (Federoff, 1996; Geller & Breakefield, 1988). HSVPrPUC contains the HSV immediate early 4/5 promoter, a multiple cloning site and SV40 A sequence and has been described previously (Paterson & Everett, 1990; Johnson *et al.* 1992; Xu *et al.* 1994; Linnik *et al.* 1995; Bergold *et al.* 1993). The RR1 cells used for packaging HSV amplicons were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (HG, 4.5 g/l), 10% FCS, 1% penicillin/streptomycin and 400 µg/ml of bioactive geneticin (G418, Gibco) at 37°C, 5% CO_2. RR1 cells are BHK cells stably transfected with the HSV IE3 gene and were obtained from Dr. Paul Johnson (Johnson *et al.* 1992). D30 EBA helper virus was prepared by growth on RR1 cells. D30EBA is a strain 17 derived IE3 mutant deleted from codons 83 to 1236 and was obtained from Dr. Roger Everett (Paterson & Everett, 1990). To package amplicon vectors, 3 X 10^6 RR1 cells were plated in media containing 10% FCS and 4 h later

were transfected by adding 40 µl of Lipofectin (Gibco-BRL), waiting 5 min and then adding the amplicon DNA solution dropwise (30 µg at 1 µg/µl in DMEM). Six hours later, plates were fed with media containing 5% FCS. Approximately 20 h after transfection, D30 EBA virus in 50-100 µl was added to achieve a multiplicity of infection (MOI) of 0.2. Five ml of complete media with 5% FCS were added to each plate after 1 h. Amplicon virus stocks were harvested 2 days later. After overnight storage at -70°C, fresh RR1 cells (4 X 10^6 cells/60 mm plate) were infected with sonicated and warmed (34°C) virus stock. Two days later, the stocks were harvested and stored for subsequent use. HSVlac virus stocks were titered by an expression assay. In brief, NIH 3T3 cells were plated (2 X 10^5 cells per well of 24 well plate) and infected with increasing volumes of an HSV amplicon virus stock in duplicate. Twenty-four h after infection, cells were fixed and stained with the chromogenic substrate 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal) using standard methods (Geller & Breakefield, 1988). The number of X-gal+ (blue) cells were counted. Titers are expressed as the number of blue forming units/ml. The D30 EBA helper virus in each stock was titered by plaque assay on RR1 cells, and HSVil2 was titered by a slot blot assay as described previously (Geschwind *et al.* 1994). For slot blot analysis, viral DNA was extracted from packaged virus by phenol/chloroform twice, ethanol precipitated with single strand calf thymus DNA as carrier, denatured at room temperature with 0.2 N NaOH, 0.5 M NaCl for ten minutes and loaded on nylon membrane with a slot blot apparatus. The membrane was then baked for 2 hours at 65°C, and probed with a [^{32}P]-labeled 435 bp SspI and PvuI fragment containing part of the β-lactamase gene from pBR322 (nucleotides 3733-4168). After stringent washing (0.1 x SSC twice for 15 minutes), blots were exposed to X-Ray film and various timed exposures taken and densitometrically scanned (LKB Ultroscan). Band densities between HSVlac and HSVil2 were compared and the titer of HSVil2 calculated from the density relative to HSVlac given that this latter amplicon was titered by an expression assay (blue forming units on NIH 3T3 cells). The titers of HSVil2 are expressed as particles/ml.

Titers of amplicon stocks: HSVlac titers were between 2 x 10^6 blue forming units/ml as titered by expression and X-gal histochemistry on NIH 3T3 cells. The HSVil2 titers, determined by slot blot (described above), were between 0.8 and 2 x 10^6 particles/ml. D30EBA titers in stocks ranged between 5 X 10^6 to 6 X 10^7 plaque forming units/ml.

Recombination for wildtype revertants was monitored by plaque assay on Vero cells and occurred at a frequency of 1 x 10^{-6}.

EXAMPLE 2

Murine hepatoma cells were transduced *ex vivo* using amplicons prepared as in example 1. Murine HEPA 1-6 hepatoma cells (ATCC, Rockville, Maryland) were maintained in DMEM + HG +10% FCS. This is a non-immunogenic hepatoma cell line (Engvall *et al.* 1977). Cells were plated at either 2 or 10 X 10^5 cells/well for all virus expression studies. In some experiments, cells were irradiated 2 h after plating and then infected with HSV amplicon stocks. In other experiments, cells were irradiated 1 h after infection with HSV amplicon stocks. Hepatoma cells were irradiated at room temperature with a 6-mV Varian CL6-100 linear accelerator at a dose-rate of 100 rads/min. To assess the rapidity of HSV amplicon gene transfer, hepatoma cells were exposed to vector stocks for either 20 or 60 min, washed extensively and cultured. After an additional 48 h, cells were histochemically stained with X-gal (HSVlac) or media assayed for IL-2 (HSVil2). In some experiments tumor cell lysates were prepared by suspension in a solution containing 0.15 M NaCl, 50 mM Tris, 1 % NP-40, 4 mM NaF, pH = 8, and assayed for IL-2. Additionally, representative samples were harvested 48 hours after treatment and viable tumor cells counted.

The results of these experiments on the efficiency of gene transfer according to the invention are summarized in Fig. 1A and B. As shown, both the HSVlac and HSV-IL-2 amplicon stocks gave maximum transfer efficiencies at an MOI of 1 or greater. In HSVlac infected cultures (Fig. 1 A), greater than 50% of the hepatoma cells expressed the reporter gene, ß-galactosidase. Fewer cells (30%) expressed ß-galactosidase when infected at an MOI of 0.5. HSVil2 infected cultures (Fig. 1 B, MOI 1.0) secreted 1,200 ± 160 ng/10^6 cells/24 hours. The immunoreactive IL-2 detected by ELISA was confirmed to be bioactive by the CML assay. Each 50 pg of immunoreactive IL-2 was equivalent to approximately 1 unit of bioactivity. The extent of gene transfer was equivalent at whether virus exposure was 20 or 60 minutes (Figs. 1C and D), indicating that virtually all infectious HSV virions adsorb to cells within 20 min. In addition, rapid gene transfer was not a

function of MOI, since expression was comparable in 20 and 60 min exposures periods at both MOIs tested (0.5 and 1.0, Figs. 1C and D).

Although IL-2 secretory rates from HSVil2-infected hepatoma cells were appreciable and in the range previously demonstrated to be immunomodulatory, it was possible that additional IL-2 might remain in an intracellular compartment. To address this issue, IL-2 measurements were made on infected cell lysates and compared with the levels found in media conditioned by these cells (Fig. 1E). The amount of IL-2 secreted in a 24 hour period was approximately 10-fold greater than the cellular content (media: 1400±100 ng/10^6 cells/24 h, lysate:100±9 ng/10^6 cells/24 h), suggesting the that the murine hepatoma cells efficiently secreted the cytokine.

Because radiation treatment of tumor cells has been viewed as an important part of producing non-dividing tumor vaccines, the affects of the timing of cell irradiation relative to HSV infection on gene transfer efficiency was investigated. As shown in Figs. 2A and B, irradiation prior to (broken lines) or just after (solid lines) HSV infection produced similar gene transfer efficiencies. Although there was a trend to higher gene transfer and expression levels in cells infected prior to irradiation, this difference was not significant. This trend towards higher gene transfer in cells infected prior to irradiation was not due to a difference in cell viability (Table 2). Of particular interest was the observation that cells irradiated at different doses secreted levels of IL-2 that were comparable to non-irradiated cells (Fig. 2C). Moreover, although irradiation affects cellular replication functions, it appears to have no affect on the biogenesis of secreted IL-2.

EXAMPLE 3

Human tumor cells were transduced *in vitro* using an amplicon containing the interleukin-2 gene produced in accordance with Example 1. This study was performed with approval and under the guidelines of the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center. Tumor biopsies of approximately 5 grams were obtained from four patients undergoing liver resection for hepatobiliary malignancies. The patient characteristics are listed in Table 1. All specimens were removed prior to any vascular interruption or Pringle maneuvers. Histologic verification of tumor was obtained in all cases. Tumor specimens were immediately placed in cold (4°C) RPMI-1640 for transport to

the laboratory. Each specimen was then minced into fine pieces and treated with 0.125% trypsin/0.125% EDTA in PBS without Ca^{++} or Mg^{++} for 5 min. The treated tumor was then teased apart and filtered through a sterile 85 μm nylon mesh into RPMI-1640 medium (4°C) containing 10% human serum. Freshly-isolated cells in suspension were irradiated at 10,000 rads at room temperature with a 6-mV Varian CL6-100 linear accelerator at a dose-rate of 100 rads/min. Aliquots of 10^6 tumor cells were then infected with HSV amplicon stocks for 20 min. Aliquots of non-irradiated cells were treated similarly and served as controls. After exposure to virus, tumor cells were washed twice and cultured at 37°C, 5% CO_2. Forty-eight h after transduction, media from each well was harvested and assayed for IL-2.

While no IL-2 was produced by any of these tumor cells prior to HSVil2 infection (Table 1), infection with HSVil2 resulted in IL-2 production by cells from all four of the tumors. In addition, as with the murine hepatoma cell lines, efficiency of gene expression was unaffected by irradiation with 10,000 rads. Finally, it is noteworthy that the entire procedure, including the radiation time, required less than 4 h, a time period that would be commensurate with intraoperative autologous vaccine generation, allowing potential reimplantation into exposed tumor sites during the same operative procedure.

EXAMPLE 4

Media and cell lysate from HSVil2-transduced tumor cells were harvested at 48 h and immediately frozen at -70°C until assay. Immunoreactive IL-2 levels were determined by standard sandwich ELISA (Biosource International, Camarillo, CA). The total IL-2 produced in the forty-eight hours of cell culture were divided by two to arrive at average production per twenty-four hours. Interleukin-2 bioactivity in the supernatant or cell lysate was also determined by assessing their ability to induce proliferation of CTLL-2 cells in a standard cell mediated lympholysis (CML) assay (Zier, 1982). Briefly, 5 X 10^5 CTLL-2 cells were mixed with serial dilutions of test samples and cultured at 37°C, 5% CO_2. After 24 h, cell viability was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml) incorporation. Recombinant human IL-2 (Chiron Corporation, Emeryville, CA) is used as an internal standard. Units are given as Cetus units.

EXAMPLE 5

To evaluation transduction efficiency, histochemical analysis was performed on tumor cells transduced with HSVlac. The cells were fixed at 48 h and histochemically stained with X-gal (Dannenberg & Suga, 1981). Briefly, plates containing transduced cells were fixed for 5 min with 1% glutaraldehyde, washed 3 times with PBS, then incubated with X-gal solution (X-gal (pH=7.4)[1 mg/ml] in PBS containing 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$-$3H_2O$). Total cells and blue cells were counted and transduction efficiency expressed as percent of total cells that were blue.

EXAMPLE 6

To determine the *in vivo* effects of tumor vaccines produced using HSV-mediated gene transfer, syngeneic C57Bl/6j mice were immunized using murine HEPA 1-6 hepatoma cells radiated with 10,000 rads and then exposed to HSVil2 at an multiplicity of infection (MOI) of 1 for twenty minutes. The hepatoma cells (10^6 cells) were washed thrice with media after the twenty minute viral exposure and immediately injected either 1) subcutaneously, 2) intraperitoneally, or 3) intrasplenicly. Animals were given either a single injection or a daily injections on three consecutive days (three injections total). As controls, animals were injected with 1) media (media-control), or 2) a similar number of radiated tumor cells exposed to HSV-lac (MOI=1), namely HSV carrying no cytokine genes (HSV-control). Animals were sacrificed three weeks later and splenocytes harvested for assessment of specific and non-specific tumor cell kill by coincubation with hepatoma for assessment of specific tumoricidal activity, K562 erythroblastic cell line for assessment of NK activity, or a syngeneic colorectal tumor cell line CO51 (ATCC; Rockville, MD) for further assessment of non-specific tumoricidal activity.

In order to determine if vaccinations with HSV-modified tumor vaccine may alter *in vivo* response to tumor, C57Bl/6j mice were immunized by intrasplenic injection with 1)10^6 radiated tumor cells exposed to HSV carrying no cytokine genes (HSV-control), or 2) 10^6 radiated, IL-2 secreting hepatoma cells. Three weeks later, the animals were injected intraportally with 10^6 replicating hepatoma cells to determine host response to tumor. Three weeks after this tumor challenge, all animals were sacrificed, and tumor growth in the liver assessed.

Splenocyte isolation was carried out as follows. Spleens were harvested from pentobarbital anesthetized animals under sterile conditions. Each spleen was placed in a petri dish containing 10 ml of PBS, brought into the hood and transferred to a new petri dish with 10 ml of RPMI + 10% FCS + 50 μg/ml gentamicin. Splenocytes were washed from the spleen by repeated injection with media. Cells will be spun (300 g, 5 min) and resuspended in 5 ml of red blood cell lysis solution (pH=7.4) (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA). After 1 min, solution were diluted with 5 ml of RPMI, 10% FCS. Cells will be spun (300 g, 10 min) and washed 2X with media. Cells were then resuspended in 30 ml of RPMI + 10% FCS + 50 μg/ml gentamicin + 30 U/ml IL-2 (Chiron Corp, Emeryville, CA) and kept in culture for 2 d prior to use. Prior to assay, cells were spun, resuspended, counted and volume adjusted to form the appropriate concentration.

The experiments summarized above examining the effects of the route and number of injections on immunization, by the subcutaneous route or intraperitoneal route, showed that three injections were necessary for specific tumor immunity. However, for the intrasplenic route, the hepatoma cell line tested elicited specific immunity with a single injection ((Figs. 3A-C, Fig. 3A presents data for HEPA 1-6 targets; Fig. 3B for K562 targets and Fig 3C for CO51 targets). This is the reason that the intrasplenic route was used for the subsequent experiment examining the effects of immunization on *in vivo* tumor growth.

Mice pretreated by intrasplenic injection of either 1) irradiated, HSV-treated tumor (HSV-control) or 2) irradiated, HSVil2 treated tumor were subsequently challenged with intraportal injection of 10^6 replicating tumor cells to determine the effects of immunization on tumor growth. Immunization using irradiated IL-2 secreting tumor cells produced by HSV-mediated gene transfer conferred *in vivo* antitumor effects. In animals treated with HSV-control, seven of the ten animals challenged with 10^6 hepatoma cells developed liver tumors, with mean tumor size being 1.5±0.4 gm (6±2% body weight). For animals pretreated with HSVil2 however, only one of ten animals developed tumor (p=0.02 vs HSV-control) with the size of that tumor being 0.2 gm (0.9 % body weight).

EXAMPLE 7

K562 or tumor cells served as targets in *in vitro* europium release cytotoxicity assays. 5 x 10^6 cells from culture were washed 2X with Buffer 1 (pH=7.4) (50 mM Hepes, 93 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$) then incubated in labeling solution (K562: 30 ml $EuCl_3$, 10 ml DTPA, 250 ml Dextran Sulfate in Buffer 1; Hep: 35 ml $EuCl_3$, 10 ml DTPA, 100 ml Dextran Sulfate in Buffer 1) for 15 min in an ice bath, mixing gently every 5 min. After 15 min, 20 ml of 100 mM $CaCl_2$ was added and the mixture incubated for 5 min. Nine ml of Repair Buffer (Buffer 1, 2 mM $CaCl_2$, 10 mM glucose) was added. Cells were spun (200 g, 10 min) and washed 4X with Repair Buffer and 3X with media. Cells then were resuspended and plated at a concentration of 5 x 10^4 cells/100 ml per well in a 96 well U-Bottom plate (Costar Corp., Cambridge, MA) containing effector cells in wells at effector to target ratios of 100:1, 50:1, 25:1, and 12.5:1. The plate was spun (10 g, 5 min), incubated (4-6 hr, 37°C), and spun (100 g, 5 min). 20 µl of supernatant were transferred to a 96 well Flat Bottom plate (Costar Corp) already containing 180 µl Delfia Enhancement Solution (Wallac Oy, Turku, Finland). The plate was read in a 1232 Delfia Fluorometer (Wallac Oy). Maximum release was measure by lysing cells with 1% Triton X. Percentage specific lysis was equal to (experimental - spontaneous release)/(maximum release+ spontaneous release) X 100. Spontaneous release varied between 5 and 15% of max.

EXAMPLE 8

HSV vectors containing the gene for either IL-2 (HSVil2) or LacZ (HSVlac) were constructed in accordance with Example 1. Twenty-five Fischer rats with bilateral flank squamous cell lung tumors were randomized to receive left flank injections of either HSVil2, HSVlac, saline or no injection on weeks 5, 7 and 9 post-implantation. Tumor volume was measured 3 times weekly for 6 weeks. There were no significant differences in tumor growth and volume among the HSVlac, saline and non-injected groups. At 6 weeks, the HSVil2 group had an 81% reduction in mean tumor volume in the injected left flank compared to controls. There was also an 88% reduction in mean tumor volume in the opposite, non-injected flank, thus indicating that *in vivo* transfection of tumor by HSV vectors containing cytokine genes is effective to stimulate a systemic antitumor response.

Four of the 5 HSVil2-treated animals were clinical responders. Staining studies for LacZ revealed transfection of tumor and surrounding stromal cells only on the treated side.

EXAMPLE 9

Murine GM-CSF, human IL-2 and LacZ genes were cloned directionally into HSVprPUC which contains the HSV immediate early 4/5 promoter, a multiple cloning site, and an SV40 A sequence, and packaged as previously described by Geller et al. (1990). RR1 cells (BHK cells stably transfected with the HSV IE3 gene) (20), along with D30 EBA helper virus (a strain 17-derived IE3 mutant deleted from codons 83 to 1236 and maintained in Dulbecco's modified Eagle medium (DME) containing high glucose [HG, 4.5 g/liter], 10% FCS, 1% penicillin/streptomycin, and 400 µg/ml of bioactive geneticin [G418; Gibco BRL, Gaithersburg, MD] at 37°C and 5% CO2) were used for packaging HSV amplicons. To package amplicon vectors, 3 × 106 RR1 cells were plated in media containing 10% FCS and transfected 4 h later by adding 40 µl of Lipofectin (Gibco), waiting 5 min, and adding amplicon DNA solution dropwise (30 µg at 1 µg/µl in DME). 6 h later, plates were fed with media containing 5% FCS. 20 h after transfection, D30 EBA virus in 50-100 µl was added to achieve an moi of 0.2. 5 ml of complete media with 5% FCS were added to each plate after 1 h, and amplicon virus stocks were harvested 2 d later. After overnight storage at 70°C, fresh RR1 cells (4×10^6 cells/60 mm plate) were infected with warmed (34°C), sonicated virus stock. 2 d later, stocks were harvested and stored for subsequent use. HSVlac stocks were titered by an expression assay using NIH3T3 cells plated (2 × 105 cells/well of a 24-well plate) and infected with increasing volumes of virus stock in duplicate. 24 h after infection, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl -D-galactoside (X-gal) using standard methods. The number of X-gal+ (blue) cells were counted, and titers were expressed as the number of blue forming units/ml. The D30 EBA helper virus in each stock was titered by plaque assay on RR1 cells, and the cytokine-containing vectors were titered by slot blot analysis. For slot blot analysis, viral DNA was extracted twice from packaged virus by phenol/chloroform, ethanol-precipitated with single-strand calf thymus DNA as carrier, denatured at room

temperature with 0.2 N NaOH, 0.5 M NaCl for 10 min, and loaded on a nylon membrane with a slot blot apparatus. The membrane was baked for 2 h at 65°C and probed with a [32P]-labeled 435 bp SspI and PvuI fragment containing part of the β-lactamase gene from pBR322 (nucleotides 3733-4168). After stringent washing (0.1× SSC 2× for 15 min), blots were exposed to x-ray film, and various timed exposures taken and densitometrically scanned (LKB Ultroscan; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Band densities and the titers of HSVil2 and HSV GM-CSF (expressed as particles/ml) calculated from the density relative to HSVlac given that this latter amplicon was titered by an expression assay, were compared. HSVlac titers were between 1-2 × 106 blue forming units/ml as titered by expression and X-gal biochemistry on NIH 3T3 cells. The HSVil2 and HSVGM-CSF titers were between 1-2 × 106 particles/ml. The ratio of D30 EBA helper virus to amplicon varied from 2:1 to 5:1. moi refers to the amplicon. Recombination for wild-type revertants was monitored by plaque assay on Vero cells and occurred at a frequency of 1×10^6.

To assess *in vitro* production of cytokines, 10^6 hepatoma cells per 2 ml were plated in six-well plates (Costar), irradiated with 10,000 rad, and rested for 1 h. Cells were then exposed to HSV-IL12, HSVGM-CSF, HSVlac, or Media for 20 min at moi's of one and two and washed 2× with media. Cell culture supernatants were harvested on days 1, 2, 4, and 7 post-exposure, and cytokine levels were measured by ELISA (IL-2, R & D Systems, Minneapolis, MN; GM-CSF, Genzyme Corp., Cambridge, MA).

As shown in Fig. 4, control cells not exposed to cytokine gene-containing vectors do not produce cytokines, and no cytokines are seen immediately after transduction with HSVil2 and HSVgm-csf and washing, indicating that proteins are not injected along with the tumor cells. Cells exposed to HSVil2 or HSVgm-csf produce nanogram quantities of these cytokines per 10^6 cells after vaccination, peaking on day 1 and decreasing thereafter.

EXAMPLE 10

Hepatoma cells in culture were irradiated with 10,000 rad, allowed to rest for 1 h, then exposed to HSVil2, HSVGM-CSF, HSVlac or media for 20 min at an moi of one. Cells were then washed 2× with media, and 10^6 cells/ 200 µl were injected intrasplenically.

An additional control group underwent injection of media alone. On day 18, half the animals in each group received either 5 × 104 U of γ-IFN i.p. or normal saline for 3 d. On day 21, all animals received a challenge of 5 × 105 hepatoma cells/200 μl intrasplenically followed by splenectomy 10 min later, allowing sufficient time for the hepatoma cells to migrate to the liver. Animals were killed 20 d later, and tumor nodules were counted. Additional animals were vaccinated, killed on d 2 and 18 post-vaccination, and heart, lung, liver, kidney and serum harvested for assessment of *in vivo* production of cytokines by ELISA.

There was no significant effect on tumor growth as a result of vaccination with irradiated cells or vaccination with irradiated cells transduced with HSVlac compared to vaccination with medium alone. As shown in Fig. 5, animals immunized with IL-2 or GM-CSF-secreting cells or pretreated with γ-IFN had significantly fewer tumor nodules that all three control groups. Combination treatment with IL-2 or GM-CSF secreting cells and pretreatment with γ-IFN was more effective than any single treatment. Complete responses were seen in 8 of 11 IL-2 animals and 4 of 12 GM-CSF animals. No animal treated with γ-IFN alone was without tumor.

EXAMPLE 11

To assess the effects of vaccination on tumor growth following a partial hepatectomy (shown to be immunosuppressive and to accelerate the growth of hepatic tumors), animals were immunized intrasplenically with hepatoma vaccines (HSVil2, HSVGM-CSF, HSVlac) produced as above. On day 18, half the animals in each group received either 5 × 104 U of IFN intraperitoneally, or normal saline for 3 d. On day 21, all animals received a challenge of 5 × 105 hepatoma cells/200 μl intrasplenically followed by splenectomy 10 min later. Half the animals in each group underwent 70% partial hepatectomy 1 h after tumor injection. One control group did not undergo vaccination or partial hepatectomy. Animals were killed 18 d after tumor challenge, and nodules were counted. In previous experiments, the number of surface nodules was shown to correlate directly with tumor volume as measured by water displacement.

As shown in Fig. 6, treatment with IL-2 or GM-CSF secreting cell lines or pretreament with γ-IFN reduced the growth of hepatectomy-induced tumors. The best

results, comparable to the results for animals with no hepatectomy, were obtained using a combination of either IL-2 or GM-CSF secreting cell lines and pretreament with γ-IFN.

EXAMPLE 12

To assess the effect of vaccination and IFN on splenocyte and Kupfer cell (KC) function, animals underwent vaccination and IFN treatment as described in Example 11, and splenocytes and KC were harvested on day 21 post-vaccination. Tumoricidal activity was assessed by mixing effectors with Europium-labeled tumor cells in an *in vitro* assay. Labeled cells were plated at a concentration of 5×10^4 cells/100 µl per well in a 96-well U-Bottom plate (Costar) containing effector cells in wells at varying effector to target ratios. The plate was spun (200 rpm, 5 min), incubated (4 h, 37°C), and respun (500 rpm, 5 min). 20 µl of supernatant were transferred to a 96-well Flat Bottom plate (Costar) already containing 180 µl/well of Delfia Enhancement Solution (Wallac Oy, Turku, Finland). The plate was read in a 1232 Delfia Fluorometer (Wallac Oy). Maximum lysis was measured by lysing cells with 1% Triton X. Percent specific lysis is equal to experimental - spontaneous release/max. release + spontaneous release × 100. Spontaneous release varied between 5 and 15% of max. Assays were performed in triplicate.

Vaccination with HSVlac or irradiated cells had no significant effect on either KC function or splenocyte activity. Splenocytes from animals vaccinated with HSVil2 or HSVgm-csf exhibited significantly greater killing of targets than splenocytes from control or γ-IFN- treated animals. γ-IFN did not appear to affect splenocyte activity. KC from rats pretreated with γ-IFN had significantly greater killing of targets than KC from controls. KC from rats vaccinated with HSVil2 also had significantly greater killing of targets than KC form controls, but not as great as KC from γ-IFN-treated rats. Vaccines secreting GM-CSF did not appear to affect KC activity.

EXAMPLE 13

Murine IL12m35, murine IL12m40, human IL2 and *LacZ* genes were cloned directionally into HSV/PRPuc and packaged as previously described. (Geller et al. (1990), Geller and Breakefield (1988), Federoff (1996). To produce HSVm75, the m35 and m40, genes were cloned directionally using appropriate restriction enzymes into HSV/PRPuc

separated by an IRES fragment. HSVPrPUC contains the HSV immediate early 4/5 promoter, a multiple cloning site and SV40 A sequence. The RR1 cells used for packaging HSV amplicons were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (HG, 4.5 g/l), 10% FCS, 1% penicillin/streptomycin and 400 µg/ml of bioactive geneticin (G418, Gibco) at 37 C, 5% CO_2. RR1 cells are BHK cells stably transfected with the HSV IE3 gene and were obtained from Dr. Paul Johnson. Johnson et al. (1992). D30 EBA helper virus was prepared by growth on RR1 cells. D30EBA is a strain 17 derived IE3 mutant deleted from codons 83 to 1236 and was obtained from Dr. Roger Everett . Paterson and Everett (1990). To package amplicon vectors, 3 X 10^6 RR1 cells were plated in media containing 10% FCS and 4 h later were transfected by adding 40 µl of Lipofectin (Gibco-BRL), waiting 5 min and then adding the amplicon DNA solution dropwise (30 µg at 1 µg/µl in DMEM). Six hours later, plates were fed with media containing 5% FCS. Approximately 20 h after transfection, D30 EBA virus in 50-100 µl was added to achieve a multiplicity of infection (MOI) of 0.2. Five ml of complete media with 5% FCS were added to each plate after 1 h. Amplicon virus stocks were harvested 2 days later. After overnight storage at -70 C, fresh RR1 cells (4 X 10^6 cells/60 mm plate) were infected with sonicated and warmed (34 C) virus stock. Two days later, the stocks were harvested and stored for subsequent use. HSVlac virus stocks were titered by an expression assay. In brief, NIH 3T3 cells were plated (2 X 10^5 cells per well of 24 well plate) and infected with increasing volumes of an HSV amplicon virus stock in duplicate. Twenty-four h after infection, cells were fixed and stained with the chromogenic substrate 5-bromo-4-chloro-3-indolyl ß-D-galactoside (X-gal) using standard methods. (Geller and Breakefield (1990)) The number of X-gal+ (blue) cells were counted. Titers are expressed as the number of blue forming units/ml. The D30 EBA helper virus in each stock was titered by plaque assay on RR1 cells, and HSVil2 was titered by a slot blot assay. For slot blot analysis, viral DNA was extracted from packaged virus by phenol/chloroform twice, ethanol precipitated with single strand calf thymus DNA as carrier, denatured at room temperature with 0.2 N NaOH, 0.5 M NaCl for ten minutes and loaded on nylon membrane with a slot blot apparatus. The membrane was then baked for 2 hours at 65 C, and probed with a [^{32}P]-labeled 435 bp SspI and PvuI fragment containing part of the β-lactamase gene from pBR322 (nucleotides 3733-4168). After stringent washing (0.1 x SSC twice for 15

minutes), blots were exposed to X-Ray film and various timed exposures taken and densitometrically scanned (LKB Ultroscan). Band densities between HSVlac and HSVil2 were compared and the titer of HSVil2 calculated from the density relative to HSVlac given that this latter amplicon was titered by an expression assay (blue forming units on NIH 3T3 cells). The titers of HSVil2 are expressed as particles/ml.

HSVlac titers were between 2 x 10^6 blue forming units/ml as titered by expression and X-gal histochemistry on NIH 3T3 cells. The HSVil2 titers, determined by slot blot (described above), were between 0.8 and 2 x 10^6 particles/ml. D30EBA titers in stocks ranged between 5 X 10^6 to 6 X 10^7 plaque forming units/ml. Recombination for wildtype revertants was monitored by plaque assay on Vero cells and occurred at a frequency of 1 x 10^{-6}.

EXAMPLE 14

Efficiency of transduction with HSVm35+HSVm40 vs. HSVm75 was assessed by measuring *in vitro* production of cytokines. To assess *in vitro* production of cytokines, 10^6 hepatoma cells per 2 ml were plated in 6-well plates (Costar), radiated with 10,000 rads and rested for 1 h. Cells were than exposed to HSVm35, HSVm40, HSVm35 + HSVm40, HSVm75, HSVlac or Media for 20 min at a multiplicity of infection (MOI) of between 1 and 4 and then washed 2X with media. Cell culture supernatants were harvested on days 1, 2, 4, 5 and 7 post-exposure, and cytokine levels were measured by ELISA specific for the heterodimeric protein.

Control cells not exposed to cytokine gene-containing vectors do not produce cytokines. IL12 production was not detected in cells transduced with either HSVm35 or HSVm40 alone. Transduction using 2 vectors produced levels of IL12 similar to transduction using a single vector carrying both genes, which peak on day 1 and decrease thereafter

EXAMPLE 15

To determine the effect of vaccination on hepatic tumor growth, hepatoma cells in culture were radiated with 10000 rads, rested for 1 h, then exposed to HSVil2, HSVm75, HSVil2 + HSVm75, HSVm35 + HSVm40, or media for 20 min at an MOI of 1-4.

Cells were washed 2X with media, and 10^6 cells/200 µl were injected intrasplenically. An additional group received 2 populations of cells: 10^6 HSVil2-transduced cells and 10^6 HSVm75-transduced cells. On day 21, *all* animals received a challenge of 5 X 10^5 hepatoma cells/200 µl intrasplenically followed by splenectomy 10 min later. This model produces uniform numbers of tumors within the liver that can be counter on day 20 after tumor challenge. Operative procedures were performed under pentobarbital anesthesia (25 mg/kg i.p.) via midline abdominal incision. Animals were sacrificed 20 days later and tumor nodules counted.

Animals immunized with cells transduced by HSVm35 + HSVm40, HSVm75 or HSVil2 had significantly fewer tumor nodules than control. Vaccination with 2 tumor cell populations, one secreting IL2 and one secreting IL12, was more effective than vaccination with a single population of cytokine-secreting cells. Vaccination with a single population of cells transduced by both HSVil2 and HSVm75 was the most effective treatment, significantly better than any single treatment or two population treatment.

EXAMPLE 16

To access the effect of vaccination on splenocyte and KC Function, animals underwent vaccination as described in Example 15, and splenocytes and KC were harvested on day 21 post-vaccination and assessed for tumoricidal activity by standard Europium-release assay. Briefly, tumoricidal activity was assessed by mixing effectors with Europium-labeled tumor cells *in vitro*. Labeled cells were plated at a concentration of 5 x 10^4 cells/100 µl per well in a 96 well U-Bottom plate (Costar) containing effector cells in wells at varying effector to target ratios. The plate was spun (200 rpm, 5 min), incubated (4 hr, 37¡C), and spun (500 rpm, 5 min). 20 µl of supernatant were transferred to a 96 well Flat Bottom plate (Costar Corp) already containing 180 µl/well of Delfia Enhancement Solution (Wallac Oy, Turku, Finland). The plate was read in a 1232 Delfia Fluorometer (Wallac Oy). Maximum lysis was measured by lysing cells with 1% Triton X-100. Percent specific lysis is equal to (experimental - spontaneous release)/(max. release + spontaneous release) X 100. Spontaneous release varied between 5 and 15% of max. Assays were performed in triplicate.

Splenocytes from animals vaccinated by either HSVil2 or HSVm75 had significantly greater killing of targets than splenocytes from animals vaccinated by radiated cells. Splenocytes from animals vaccinated by cells transduced by HSVm75 *and* HSVil2 had significantly greater killing of targets than splenocytes from animals vaccinated by a single cytokine at an effector to target ratio of 100:1.

KC from rats vaccinated with HSVil2 or HSVm75 had significantly greater tumoricidal activity than KC from controls ($p < 0.05$) at effector to target ratio of 50:1. KC from animals vaccinated by cells transduced by HSVm75 *and* HSVil2 had significantly greater killing of targets than KC from animals vaccinated by a single cytokine at an effector to target ratio of 100:1.

EXAMPLE 17

Human ICAM-1 and E. coli β-galactosidase cDNA was directionally cloned into HSVPrPuc (HSVhicam1 and HSVlac respectively) which contains the HSV immediate early 4/5 promoter, a multiple cloning site, and an SV40 A sequence, and packaged as previously described in Example 1. RR1 cells (BHK cells stably transfected with the HSV IE3 gene), along with D30 EBA helper virus (a strain 17-derived IE3 mutant deleted from codons 83 to 1236 and maintained in Dulbecco's modified Eagle medium (DME) containing high glucose [HG, 4.5 g/liter], 10% FCS, 1% penicillin/streptomycin, and 400 μg/ml of bioactive geneticin [G418: Gibco BRL, Gaithersburg, MD] at 37°C and 5% CO2) were used for packaging HSV amplicons.

To package amplicon vectors, 3 x 10^6 RR1 cells were plated in media containing 10% FCS and transfected 4 hours later by adding 40 μl of Lipofectin (Gibco), waiting 5 min, and adding amplicon DNA solution dropwise (30 μg at 1 μg/μl in DME). Six hours later, plates were fed with media containing 5% FCS. Twenty hours after transfection, D30 EBA virus in 50-100 μl was added to achieve a multiplicity of infection (MOI) of 0.2. Five ml of complete media with 5% FCS were added to each plate after 1 hours, and amplicon virus stocks were harvested 2 days later. After overnight storage at 70°C, fresh RR1 cells (4 x 10^6 cells/60 mm plate) were infected with warmed (34°C), sonicated virus stock. Two days later, stocks were harvested and stored for subsequent use. HSVlac stocks were titered by an expression assay using NIH3T3 cells plated (2 x 10^5

cells/well of a 24-well plate) and infected with increasing volumes of virus stock in duplicate. Twenty four hours after infection, cells were fixed and stained with 5-bromo-4-chlor-3-indolyl Beta-D-galactosidase (X-gal) using standard methods. The number of X-gal+ (blue) cells were counted, and titers were expressed as the number of blue forming units/ml.

The D30 EBA helper virus in each stock was titered by plaque assay on RR1 cells, and the cytokine-containing vectors were titered by slot blot analysis. For slot blot analysis, viral DNA was extracted twice from packaged virus by phenol/chloroform, ethanol-precipitated with single-strand calf thymus DNA as carrier, denatured at room temperature with 0.2 N NaOH, 0.5 M NaCl for 10 minutes, and loaded on a nylon membrane with a slot blot apparatus. The membrane was backed for 2 hours at 64°C and probed with a [32p]-labeled 435 bp SspI and PvuI fragment containing part of the Beta-lactamase gene from pBR322 (nucleotides 3733-4168). After stringent washing (0.1 x SSC 2x for 15 min), blots were exposed to x-ray film, and various timed exposures taken and densitometrically scanned (LKB Ultroscan: Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Band densities and the titers of HSVhicam1 (expressed as particles/ml) calculated from the density relative to HSVlac given that this latter amplicon was titered by an expression assay, were compared . HSVlac titers were between 1-2 x 10^6 blue forming units/ml as titered by expression and X-gal biochemistry on NIH3T3 cells. The HSVhicam1 titers were between 1-2 x 10^6 particles/ml. The ratio of D30 EBA helper virus to amplicon varied from 2:1 to 5:1. MOI refers to the amplicon. Recombination for wild-type revertants was monitored by plaque assay on Vero cells and occurred at a frequency of 1 x 10^{-6}.

EXAMPLE 18

The tumor cell line Morris Hepatoma McA-RH7777 (ATCC CRL 1601) was maintained in culture (DME, 6.25% FCS, 20% Horse serum, 2mM L-Glutamine) and periodically implanted into buffalo rat flanks to ensure tumorigenicity. This cell line was tested to be free of mycoplasma and viral infection.

Hepatoma cells from culture were radiated with 10,000 rads and rested for 1 hour. Cells were then exposed to HSVhicam1, HSVlac or nothing at an MOI of 1 for 20

minutes at 37°C. Cells were then washed with media twice and maintained in culture until analysis. To assess the cell surface expression of hICAM1, cells were harvested at 1, 2, 5 and 7 days after transduction and washed twice with HBSS containing 10mM HEPES. Separate aliquots of cells were then incubated on ice for 20 minutes with anti-human ICAM-1 (Clone MEM111, Caltag, Burlingame, CA) and anti-rat ICAM-1 (Clone 1A29, Caltag, Burlingame, CA) antibodies conjugated to PE or FITC. Additional aliquots of cells were incubated with isotype controls (Caltag, Burlingame, CA) to account for nonspecific binding of antibodies. Cells were then analyzed with a FACscanner (Becton Dickinson) for the presence of human and rat ICAM.

With PE labeling, greater than 90% of normal untreated rat hepatoma cells expressed rat ICAM on the cell surface with mean fluorescent intensities ranging from 200 to 288. There was no difference in rat ICAM expression between transduced and non transduced cells. Cells transduced with HSVlac or nothing had no detectable surface human ICAM-1. Flow cytometric analysis of rat hepatoma cells transduced with HSVhicam1 showed that a 20 minute exposure, at an MOI=1 resulted in high level expression of human ICAM on the surface of tumor cells. Peak cell surface positivity for human ICAM-1 was found 24 hours after transduction and tapered off by 1 week (Percent of cells positive for hICAM1 was 25%, 16%, and 9% on days 1, 2 and 5 post transduction). Mean fluorescent intensity of human ICAM-1 on HSVhicam1-transduced cells was 450, 271, and 124 on days 1, 2 and 5 respectively. On day 7 post transduction with HSVhicam1, cell viability was limited, but approximately 4% of viable cells were positive for surface hICAM1.

Fig. 7 illustrates the quantitation of soluble human ICAM found in cell culture supernatants of transduced cells. No soluble human ICAM was detectable in supernatants of cells transduced with HSVlac or nothing. Levels in supernatants of transduced cells peaked at 48 hours after transduction and approached the level of detection by day 7.

EXAMPLE 19

To determine if ICAM-1 transduced hepatoma cells bound lymphocytes more avidly, a modification of previously reported adhesion assays (Miki, et al., 1993) was performed. Briefly, hepatoma cells were radiated with 10,000 rads, exposed to

HSVhicam1, HSVlac or nothing for 20 minutes at 37°C and washed with media twice. Cells were then plated in nearly confluent monolayers in 96 well plates. Splenocytes were harvested from normal Buffalo rats one day prior to each assay and cultured overnight in Complete RPMI (.01 mM NEAA, 1 mM NaPyruvate, 2 mM L-Glutamine, 50 μM 2-ME, Pen/Step) containing 10% FCS, 50 U/ml IL2 (Chiron Corporation, Emeryville, CA), 5 μg/ml Con A (Sigma, St. Louis, MO), and 50 ηg/ml PMA (Phorbol 12-Myristate 13-Acetate) (Sigma, St. Louis, MO). On the day of the assay, nonadherent splenocytes were harvested at a concentration of 10^6/cc, and labeled with MTT (5 mg/ml PBS) in a v:v ratio of 3:1 (splenocytes:MTT). Splenocytes were incubated with MTT for 6 hours at 37°C with gentle agitation every 30 minutes. Labeled lymphocytes were then plated at a concentration of 10^6/100μl in the wells containing the hepatoma targets. The cells were then co-incubated at 37°C for 30 minutes. Nonadherent splenocytes were then gently washed off with PBS. Adherent lymphocytes were lysed with DMSO and read by spectrophotometry at 570 ηm. Representative wells were used to count the number of hepatoma targets present for each experimental group. Additional labeled splenocytes were plated at varying concentrations, lysed and read by spectrophotometry in order to create a standard curve for the number of splenocytes per well. An adhesion index calculated as the number of adherent lymphocytes per hepatoma target cell and the mean of 8 wells was recorded.

In order to determine if hICAM1 gene transfer would alter lymphocyte binding by tumor, an *in vitro* lymphocyte binding assay was used. There was a significant increase in the number of adherent lymphocytes per hepatoma target cell in wells containing HSVhicam1-transduced cells compared to lac-transduced and untreated cell (Fig. 8). This doubling of lymphocyte binding was statistically significant ($p<0.05$).

EXAMPLE 20

In order to determine if transduction of hepatoma cells with the ICAM-1 gene altered *in vitro* growth properties, cell proliferation assays were performed. Replicating rat hepatoma cells were exposed to HSVhicam1, HSVlac or nothing at an MOI of 1 for 20 minutes at 37°C. Cells were then plated in 24 well plates at a concentration of 10^4 viable cells/ml/well. Cells were harvested by trypsin disaggregation at 1, 2 and 4 days

after plating and counted by trypan blue exclusion. The mean count of 8 wells per time point was compared. Cells transduced with HSVhicam1 grew similarly in culture compared to HSVlac-transduced cells and untreated cells, indicating that changes in *in vivo* tumor growth (see example 21) cannot be accounted for by changes in intrinsic growth rate of the modified tumor.

EXAMPLE 21

Male Buffalo rats (Harlan Sprague Dawley) were housed 2 per cage in a temperature (22°C) and humidity controlled environment and were given water and standard rat chow (PMI Mills, St. Louis, MO) *ad libitum*. They were maintained in 12 hour light/dark cycles. All surgical procedures were carried out through a midline laparotomy under i.p. pentobarbital (50mg/kg) anesthesia. For major abdominal operations, 3 ml of 0.9% saline was administered i.p. for resuscitation post operatively. All animals received care under approved protocols in compliance with Memorial Sloan-Kettering Cancer Centers Institutional Animal Care and Use Committee guidelines.

Tumorigenicity experiments

In order to analyze the effects of ICAM-1 overexpression on the *in vivo* growth characteristics of hepatoma cells, flank tumorigenicity experiments were performed. Animals (n=5 per group) were randomized to receive subcutaneous left flank injections of 10^6 viable rat hepatoma cells transduced with HSVhicam1, HSVlac or nothing (MOI of 1). On the opposite right flank, all animals received subcutaneous flank injections of 10^6 viable non-transduced cells. Animals were weighed and tumors measured with external calipers twice weekly. Tumor measurements were made in two perpendicular dimensions and averaged. Tumor volume was calculated using the equation $4/3\pi r^3$.

There was significantly decreased tumor growth in the left flanks of animals injected with HSVhicam1-transduced cells compared to controls (Fig. 9). Tumor volumes at the termination of the experiment were compared. Tumors transduced with HSVhicam1 had a significantly ($p<0.05$) smaller volume (1,397 +/- 1296 mm^3) compared to tumors transduced with HSVlac (7,109 +/- 2118 mm^3) and untreated tumors (13,556 +/- 3354 mm^3). On the contralateral untreated side, all groups had progressive tumor growth that was not significantly different.

Immunohistochemistry

In order to assess potential immunologic mechanisms of tumor regression, Immunohistochemical analysis of cell infiltrates in tumors was carried out. Animals from additional tumorigenicity experiments had tumors excised at 1 week and 3 weeks after injection of cells (n=5 per time point) and placed immediately in 10% buffered formalin. Twenty four hours later, tumors were embedded in paraffin using standard techniques. Five μm sections were made. Hematoxylin and Eosin staining was performed using standard techniques. The following antibodies were used for immunohistochemical analysis; mouse monoclonal anti-rat CD4 (IgG_1, clone W3/25, Serotec, Oxford, England), mouse monoclonal anti-rat CD8 (IgG_1, clone OX-8, Caltag, Burlingame, CA), and mouse monoclonal anti-rat 1-A (IgG_1, clone OX-6, Serotec, Oxford, England) which recognizes rat MHC Class II. The secondary antibody used was Biotinylated anti-mouse IgG, rat adsorbed (Vector, Burlingame, CA). Slides used for CD4 and CD8 staining were pretreated with 1mM EDTA (ph 8) in a microwave for 10 minutes. For MHC II staining, slides were pretreated for 10 minutes with a 0.05% Protease XXIV (Sigma, St. Louis, MO) in Tris-HCl buffer, ph 7.6. Endogenous peroxide was then quenched with a five minute incubation in 3% H_2O_2. After washes with PBS, slides are then placed in 0.05% bovine serum albumin for 1 minute. Slides were then dried and whole horse serum applied at a 1:20 dilution in 2% bovine serum albumin and incubated for 10 minutes. Serum was then suctioned off and 150 μl of primary antibody applied. The primary antibody was incubated for 16 - 18 hours at 4° C in a humidity chamber. After PBS washes, secondary antibody was applied to the slides at a 1:500 dilution in 1% bovine serum albumin and incubated for 60 minutes at room temperature in a humidity chamber. Slides were then washed in PBS and peroxidase-conjugated streptavidin was applied at a dilution of 1:500 in 1% bovine serum albumin. Slides were then washed with PBS and transferred to a bath of 0.06% diaminobenzidine (Sigma, St. Louis, MO) for 5 to 15 minutes. Slides were then washed in water and decolorized with 1% acid alcohol and blue in ammonia water. Dehydration with ethanol and xylene were carried out with standard techniques and slides were mounted with Permount (Fisher, Pittsburgh, PA) mounting media.

A single pathologist blinded to the experiment reviewed slides and graded them in the following way. Tumor cells were assessed for the presence or absence of MHC

II staining. The degree of tumor infiltration with MHC II staining non-tumor cells was graded from 1 to 4. The degree of infiltration of tumors with the total amount of CD4 and CD8 positive lymphocytes was graded from 1 to 4. The relative percentage of CD4 and CD8 positive cells was then assessed and expressed as a ratio. Rat splenic tissue was used as a positive control for each experiment.

The amount of infiltration of tumors with both CD4 and CD8 positive T lymphocytes did not differ between treatment groups at 1 and 3 weeks. The ratio of CD4 to total CD4 and CD8 positive T cells did not differ between groups at 1 week, but at 3 weeks, there was a significant increase in this ratio in the HSVhicam1-treated animals compared to HSVlac and untreated animals (0.42 vs. 0.25 and 0.24, $p < 0.05$). There was no significant difference in the degree of infiltration of tumors with MHC II staining immune cells between treatment groups at 1 and 3 weeks. Tumor cells did not stain positively for MHC II expression in any case.

EXAMPLE 22

In order to determine whether previous exposure to ICAM-1 transduced hepatoma cells would protect against future challenges with the parental tumor, vaccination experiments were performed. Whole tumor cell vaccines were prepared as follows. Rat hepatoma cells were radiated with 10,000 rads, exposed to HSVhICAM1, HSVlac or nothing at an MOI of 1 for 20 minutes at 37°C and washed twice with media. Animals (n=19 per group) were then randomized to receive either cell type by intrasplenic injections of 10^6 cells in 200µl of media on day 1. Control animals received 200µl of media intrasplenically. Three weeks after vaccination, animals were challenged with 5×10^5 replicating hepatoma cells by intrasplenic injection. After 10 minutes, a splenectomy was performed in all animals. Three weeks after challenge, animals were sacrificed and liver surface tumor nodules counted. Body weights were recorded and grooming habits monitored twice a week throughout the experiment.

Throughout the experiment, there was no difference in weight gain in all treatment groups and all animals maintained normal grooming habits. As illustrated in Fig. 10, there was significantly decreased uptake and growth of hepatic metastases in animals vaccinated with HSVhicam1 cells compared to all controls ($p \leq 0.05$). There was no

difference between animals vaccinated with HSVlac-transduced cells, radiated cells alone or media.

EXAMPLE 22

The coding sequences for human B7.1 or human RANTES were cloned into the polylinker region of the pHSVPrPUC plasmid. To form the HSV-B7.1 amplicon, pBJ.huB7.1 plasmid (kindly provided by Dr. Lewis Lanier, DNAX, Palo Alto, CA) was digested with HindIII and was filled in to generate a blunt end and. Subsequently, this plasmid was digested with Xba1. A The HindIII blunt/Xba1 fragment encoding the for the human B7.1 cDNA was gel purified and used as insert in the ligation with the vector. The HSV amplicon vector pHSVPrPUC plasmid was digested with EcoR1 and filled in with Klenow to make a blunt end, followed by Xba1 digestion. The EcoR1blunt/Xba1 vector fragment was gel purified and ligated with the insert. The constructed amplicon plasmid was analyzed for the orientation of the coding sequences of huB7.1 with respect to the HSV-1 IE4/5 promoter, and the amplicon used in the generation of the HSVB7.1 amplicon virus.

To form the HSV-RANTES amplicon, SK+pBS-RANTES plasmid (kindly provided by Dr. Tom Schall, ChemoCentryx, MountainView, CA) was partially digested with Kpn1 followed by digestion with Xba1. The Kpn1/Xba1 fragment encoding human RANTES cDNA was gel purified and used as insert in the ligated to the HSV amplicon vector pHSVPrPUC plasmid digested with Kpn1 and Xba1. Orientation of the coding sequences for huRANTES with respect to the HSV-1 IE4/5 promoter was verified, and the amplicon used in the generation of the HSVrantes amplicon virus. The HSV amplicons are shown schematically in Fig.11.

EXAMPLE 23

Amplicon DNA was packaged into HSV-1 particles by transfecting 5 μg of plasmid DNA into RR1 cells with lipofectamine as recommended by the manufacturer (GIBCO-BRL). Following incubation for 24 hours the transfected monolayer was superinfected with the HSV strain 17, IE3 deletion mutant virus D30EBA (Paterson et al., 1990) at a multiplicity of infection (MOI) of 0.2. Once cytopathic changes were observed

in the infected monolayer, the cells were harvested, freeze-thawed, and sonicated using a cup sonicator (Misonix, Inc.). Viral supernatants were clarified by centrifugation at 5000g for 10 min prior to repeat passage on RR1 cells. This second viral passage was harvested as above and concentrated overnight by ultracentrifugation in a 25% sucrose gradient as previously described (Tung et al., 1996). Viral pellets were resuspended in PBS (Ca2+ and Mg2+ free) and stored at -80°C for future use. Stocks were titered for helper virus by standard plaque assay methods. Amplicon titers were determined as follows: NIH 3T3 cells were plated in a 24-well plate at a density of $1x10^5$ cells/well and infected with the virus. Twenty-four hours after viral infection the monolayers were washed twice in PBS and either fixed with 4% paraformaldehyde and stained by X-gal histochemistry (5mM Potassium Ferricyanide; 5mM Potassium Ferrocyanide; 0.02% NP-40; 0.01% sodium deoxycholic acid; 2 mM $MgCl_2$ and 1mg/ml Xgal dissolved in PBS) or harvested for total DNA using lysis buffer (100 mM NaCl, 10mM Tris, pH 8.0, 25 mM EDTA, 0.5% SDS) followed by subsequent phenol/chloroform extraction and ethanol precipitation. PCR was performed on duplicate samples using primers corresponding to the β-lactamase gene present in the amplicon plasmid under the following conditions: 94°C, 2 min; then 20, 23 or 26 cycles of 94°C (30 sec), 58°C (30 sec), followed by 72°C (7 min). PCR products from early and late cycles were run on a 1% ethidium bromide gel, and the 450 bp band intensities were assessed using the FOTODYNE FOTO/ECLIPSE™ system (Fotodyne, Inc, Hartland, WI) and COLLAGE™ Image Analysis Software. HSVB7.1 and HSVrantes titers were estimated by comparison with HSVlac virus as standards. Plaque forming unit (pfu/ml) and amplicon (bfu/ml) titers obtained from these measurements were used to calculate amplicon titer and thus standardize experimental viral delivery. Amplicon titer in the different virus preparations ranged from 1-10 X 10^7 bfu/ml and the helper titers were in the range of 5-15 X 10^7 pfu/ml.

EXAMPLE 24

EL4 cells were infected *in vitro* either with HSVB7.1, or HSVlac amplicon virus at an MOI of 0.5-1-5 pfu per cell. Specifically, 10^6 EL4 cells were adsorbed with the amplicon virus in a volume of 0.5ml at 37°C, 5% CO_2 for 4 hours. At the end of 4 hours, 0.5ml of fresh ID-10 medium was added and incubation continued for another 12 hours. The

infected cells were harvested at the end of 16 hours and 10^6 cells in 0.1ml of chilled PBS were stained with 1:10 diluted phycoerythrin (PE) conjugated anti-B7.1 antibody (anti-CD80 PE, Becton-Dickinson) for 30 minutes at 4°C. Uninfected EL4 cells (as negative control), or EL4 stably expressing B7.1 (EL4-B7.1 as cells as positive control) were also stained simultaneously with the anti-CD80 PE antibody. The stained cells were analyzed by flow cytometry using an EPICS flow cytometry instrument.

Control uninfected EL4 cells or EL4 cells infected with HSVlac were negative for the B7.1 expression (Figs. 12A&B). In contrast, approximately 95% of EL4 cells infected at an estimated MOI of 1 stained positively for B7.1 expression (Fig. 12C). On a per cell basis, HSV-B7.1 amplicon virus infected cells showed significantly higher levels of B7.1 expression than those observed for EL4-B7.1 cell line established by retroviral transduction. Expression of B7.1 in HSVB7.1 infected cells was maintained for up to 60 hours post-infection.

EXAMPLE 25

The bioactivity of HSV vector-expressed B7.1 was studied in an *in vitro* proliferation assay. Murine T-cells were enriched using a murine T- cell enrichment column (R&D Systems). 10^5 T-cells were incubated in the presence of $5x10^4$ gamma-irradiated stimulator cells. EL4 or CHO cells infected with HSV- B7.1, were used as stimulator cells. Retrovirally transduced EL4-B7.1, or CHO-B7.1 (kindly provided by Dr. Peter Linsley) were used as a positive control for B7.1 expression and parental EL4 or CHO cells served as a negative controls. Stimulator cells were irradiated to a total of 7500 rads using a Cesium-gamma source. Either anti-CD3 antibody (2C11) used as a (2C11)1:50 dilution of the hybridoma cell culture supernatant , or phorbol myristate (10ng/ml) with ionophore (0.1ng/ml) were added and the cells were cultured for 3 days at 37OC in 5% CO2 incubator. To assay for proliferative responses in these stimulated cells, triplicate cultures were labeled for 16 hours with 1µCi ^{3}H-thymidine (NEN, 2Ci/mmol, 1µCi/ 0.2 ml, final concentration). Cells were harvested on glass fiber filters using a cell harvester (Packard Instruments) and the incorporated 33H-radioactivity was measured using a beta-counter (Packard Instruments). Results are expressed as the mean (of triplicate cultures) +/-with the standard deviation. T-cell proliferation index (normalized cpm) was

determined as the ratio of ^{3}H-thymidine incorporated in the stimulated versus unstimulated control cultures.

When stimulated with anti-CD3 antibody (2C11) or a mixture of phorbol myristate acetate (PMA) and ionophore to provide 'signal one,' a significant proliferative response was observed for T-cells cocultured with HSVB7.1, but not HSVlac infected stimulator cells. The B7.1-dependent T- cell proliferative response observed with the HSVB7.1 infected EL4 cells was comparable to that seen with the retrovirally transduced control stimulator cells EL4-B7.1 or CHO-B7.1.

EXAMPLE 26

EL4 cells were infected with HSVrantes or HSVlac amplicon at an MOI of 1. EL4 cells at $1X10^6$ were adsorbed with the amplicon virus in a volume of 0.5ml at 37°C, 5% CO^2 for 4 hours, then 0.5ml of fresh medium was added and incubation continued for another 20 hours. Cell culture supernatants were harvested at the end of 24 hours and supernatants tested for RANTES in a sandwich ELISA using anti-RANTES antibody (R&D Systems) for RANTES capture of RANTES in the culture supernatants and biotinylated anti-RANTES (R&D Systems) for detection followed by alkaline phosphatase-conjugated avidin. Para-Nitrophenyl phosphate was used as a substrate and absorbance developed color read at 405nm was read in a BIORAD ELISA reader. Serial two fold dilutions of standard recombinant human-RANTES (R&D Systems) in duplicates were run in parallel to quantitate the amount of RANTES in the culture supernatant of infected cells.

In uninfected EL4 cells or cells transduced with HSVlac, no detectable RANTES secretion was observed in culture supernatants. Cells infected with HSVrantes at an MOI of 0.5 produced 3.1ng of RANTES/ml/24 hours/10^6 cells. The observed levels of RANTES were higher than those measured in pooled G418 selected retrovirally transduced EL4-RANTES cells which secreted RANTES at a concentration of 1.45ng/ml/24 hours/106 cells.

EXAMPLE 27

Adult C57BL6 (H-2^b) female mice (8 weeks old) were obtained from Charles River Laboratories (MA) and maintained at the Animal Facility, University of Rochester

Medical Center. The mice were handled under an approved laboratory animal handling and care protocol. Mice (6-12 per group) were shaved on the dorsal side of the hind limb and were inoculated subcutaneously (sc) with 10^6 viable EL4 cells infected *ex vivo* with HSVB7.1, HSVrantes, or HSVlac amplicon virus, or with uninfected EL4 cells. In some experiments 10^6 uninfected EL4 cells were inoculated sc. contralaterally on the other hind limb at the same time. Tumor growth was measured every 2-3 days using a caliper and size reported in millimeters diameter (mm). Animals were sacrificed when the tumor size reached 22-23mm.

The results of these experiments on growth of HSV-infected EL4 cells and on contralateral EL4 tumors are summarized in Table 3 and Figs. 13A and 13B. On day 20, complete regression of tumor was noted in 3/6 mice inoculated with EL4-HSVB7.1 transduced EL4 cells. Two of six mice inoculated with HSVrantes-transduced EL4 cells also showed initial tumor growth followed by complete regression in mice inoculated with EL4 infected with HSV-RANTES. When EL4 cells were infected with both HSVB7.1 and HSVrantes, 5/6 mice showed complete regression following initial tumor growth. Control EL4 cells or EL4 cells infected with the HSVlac vector grew tumor in 100% of the mice (6/6). Stably transduced EL4-B7.1 cells showed no evidence of tumor growth in all mice by day 20. These results support the conclusion that HSVB7.1 or HSVrantes amplicon infected cells were rejected due to a tumor specific immune response.

Similar results were observed in the experiment to evaluate whether inoculation of HSV vector transduced cells would inhibit growth of concurrent contralaterally inoculated parental non-transduced EL4 cells. In 3/5 mice, regression of *ex vivo* HSVB7.1 infected EL4 tumor was concordant with regression of the contralateral EL4 tumor (Fig. 13A). Both HSVlac infected EL4 cells and contralateral parental EL4 cells developed into tumor in 5/5 animals studied (Fig. 13B). These data support the conclusion that systemic tumor specific immunity to parental EL4 cells had developed in a subset of mice inoculated with EL4-HSVB7.1 transduced EL4 cells.

To test the efficacy of HSVB7.1 and HSVrantes on pre-established tumors using intratumoral inoculation of the HSV amplicons, 10^6 viable EL4 cells were inoculated sc. on the dorsal side of the shaved hind limb and the tumor allowed to grow to a size of 5-6mm (6-7 days). At this point the mice were grouped and either HSVB7.1, HSVrantes,

HSVB7.1 + HSVrantes, or HSVlac amplicon virus diluted in PBS to a concentration of $2x10^6$ amplicon containing virus particles in 50 µl was inoculated intratumorally (10-12 mice/group). Control animals with pre-established EL4 tumor received only the diluent PBS. A second inoculation of the HSV amplicons was given on day 14, and the tumor growth was measured every 2-3 days. Tumors were allowed to grow to a maximal size of 22-23 mm size at which point the animals were sacrificed.

Complete tumor regression was observed in 17/26 mice injected with HSVB7.1 vector alone, in 11/22 mice injected with HSVrantes, and in 23/26 mice injected with the combination of HSVB7.1 and HSVrantes. Results of three independent experiments yielded similar results as summarized in Table 4.

To determine whether regression of tumor correlated with the development of systemic and memory T-cell immunity, mice manifesting complete tumor regression were rechallenged with parental EL4 cells in the on the other hind limb contralateral to the primary inoculation. All mice the rechallenged with parental EL4 cells showed no tumor growth (Table 4), thus indicating that tumor specific immunity was established by the antecedent direct intratumoral delivery of HSVB7.1 and/or HSVrantes into pre-established tumors.

EXAMPLE 28

To examine the induction of CTL responses in mice transduced intratumorallu with the HSV amplicon vectors, splenocytes from the mice of Example 27 were evaluated. Spleens were harvested from C57BL/6 mice which had been inoculated with EL4 cells and injected intratumorally with either HSVB7.1 or HSVrantes alone or in combination. Control splenocytes were obtained from mice which were inoculated intratumorally with HSVlac virus or mice with PBS diluent alone. Splenocytes were prepared according to standard procedures and red blood cells lysed using AKC lysis buffer. To obtain cytolytic T-cells, splenocyte cell suspensions (2x1066/ml in RP-10) were cultured together with gamma-irradiated (7500 rads) EL4 cells ($0.5x10^6$ cells/ml) in a 25 cm^2 flask at 5% CO_2, 37°C for 6 days. These *in vitro* cocultured splenocytes were then used as effector cells in the CTL assays. On the day of assay, EL4 target cells were washed with PBS and resuspended in RP10 medium (0.1ml) at a concentration of 1.5- $2x10^6$

cells/ml and $Na^{51}CrO_4$ (NEN, 100 μCi; stock concentration 1 mCi/ml) added for 90 minutes at 37°C. These cells were washed three times with PBS, resuspended in 1 ml RP-10 and viable cell count taken using a haemocytometer. ^{51}Cr-labeled target cells (10^4 cells/0.1 ml) were added to the wells of a V-shaped 96 well plate, and three-fold serial dilutions of effector cells were made in triplicate, resulting in final effector-target cell ratios (E:T ratios) of 100:1, 33:1, 11:1 3:1, and 1:1. Spontaneous release of radioactivity from labeled target cells was measured by culturing the target cells with medium alone in six wells. Total release of radioactivity was determined by lysing the target cells with 2% Triton-X 100 detergent. Plates were spun at 1K for 2 minutes and incubated for 4 hrs at 37°C, 5% CO_2. The plates were then centrifuged at 2K for 4 minutes and half of the culture supernatant (100μl) was counted for ^{51}Cr release in a gamma counter (Packard Instrument). Mean values are calculated for the replicate wells and the results are expressed as % specific lysis according to the formula:

$$\% \text{ specific lysis} = 100 \text{ X } \frac{\text{experimental counts} - \text{spontaneous counts}}{\text{total counts} - \text{spontaneous counts}}$$

The mean spontaneous release for virus-infected and uninfected controls averaged between 10 to 20% of the total counts.

Significant specific CTL activity was seen in splenocytes from mice receiving HSVB7.1 or HSVrantes alone or in combination (Figs. 14A-D) CTL responses were only seen in mice in which EL4 tumor regressed after direct delivery of the HSVB7.1 and/or HSVrantes amplicons into pre-established tumor. Levels of CTL activity were greater in mice which received both the HSVB7.1 and HSVrantes vectors. The highest levels of CTL activity were observed in mice which had been rechallenged with the parental EL4 cells.

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Table 1: Efficiency of IL-2 Secretion from Human Tumor Cells Transduced with HSVil2

Patient	Diagnosis: Clinical	Diagnosis: Histologic	Radiation	MOI: 0	MOI: 0.5	MOI: 1	MOI: 2
1	Met Colorectal Ca	Moderately differentiated	No	0	580±40	6400±200	10700±70
		Adenocarcinoma	Yes	0	334±4	5500±100	10500±50
2	Hepatoma	Clear cell	No	0	2100±10	2600±20	5900±70
		adenocarcinoma	Yes	0	580±40	2490±40	6450±70
3	Gallbladder Ca	Moderately differentiated	No	0	ND	12500±700	ND
		Adenocarcinoma	Yes	0	ND	4800±100	ND
4	Hepatoma	Poorly differentiated	No	0	ND	17500±500	ND
		Adenocarcinoma	Yes	0	ND	19300±600	ND

ND, not determined. Values are mean levels of samples transduced in quadruplicate ± SEM. Levels are pg/10^6 cells/24 hours.

Table 2. Effect of timing of irradiation and HSV exposure on cell viability. Hepatoma cells were either exposed to radiation (10,000 rads) followed by a 20 minute exposure to HSV (Rad/HSV), or exposed to HSV for 20 minutes followed by irradiation (10,000 rads) (HSV/Rad). Cells (5 X 10^5 cells were then plated and left in culture for 48 hours. Non-viable cells were washed off before harvesting cells for counting. In addition, harvested cells were verified to be viable by trypan blue exclusion. Comparisons were by student's t-test.

MOI	**Rad/HSV (X 10^5 cells)**	**HSV/Rad (X 10^5 cells)**	**p**
0	2.1±0.1	1.8±0.2	0.1
0.5	2.0±0.1	1.8±0.2	0.2
1.0	1.8±0.1	1.5±0.1	0.2

Table 3 Tumor growth of EL4 cells infected *ex vivo* with HSV amplicons. EL4 cells were infected *in vitro* with HSV amplicon virus and maintained in culture for 8 hours. 10^6 viable HSV amplicon infected EL4 cells were inoculated s.c. in mice and tumor presence at one month recorded.

HSV amplicon	# of mice with tumor/ # of mice inoculated
HSV-B7.1	3/6
HSV-RANTES	4/6
HSV-B7.1 & HSV-RANTES	1/6
HSV-LacZ	6/6

Table 4 Intratumoral delivery of HSV amplicons into pre-established EL4 tumors. EL4 cells were inoculated s.c. in mice and tumors allowed to develop to a 5-6 mm diameter. HSV amplicon virus was inoculated in two doses, on days 7 and 14, and tumor growth monitored and recorded after one month. The values reported correspond to the number of mice with tumor / total number of mice.

HSV amplicon	Primary Tumor Growth	Tumor Growth Following Rechallenge
Experiment # 1		
HSVB7.1	**1/4**	**0/3**
HSVB7.1 + HSVrantes	**0/4**	**0/4**
HSVlac	**4/4**	
Experiment # 2		
HSVB7.1	**4/10**	**0/6**
HSVrantes	**5/10**	**0/5**
HSVB7.1 + HSVrantes	**1/10**	**0/9**
HSVlac	**5/5**	
Experiment # 3		
HSVB7.1	**4/12**	**0/4**
HSVrantes	**6 /12**	**0/4**
HSVB7.1 + HSVrantes	**2/12**	**0/6**
HSVlac	**5/5**	

CLAIMS

1. A method for production of an autologous vaccine to tumor cells comprising transducing the tumor cells with one or more species herpes simplex virus amplicon containing the gene for an immunomodulatory protein and at least one additional therapeutic gene to provide transient expression of the immunomodulatory protein and the therapeutic gene product by the cells.

2. The method according to claim 1, wherein the tumor cells are transduced with the herpes simplex amplicons *ex vivo*.

3. The method according to claim 1, wherein the tumor cells are transduced with the herpes simplex cell *in vivo*.

4. A method for inducing a protective immune response to tumor cells in a patient comprising the step of transducing the tumor cells with one or more species herpes simplex virus amplicon containing the gene for an immunomodulatory protein and at least one additional therapeutic gene to provide transient expression of the immunomodulatory protein and the therapeutic gene product by the cells.

5. The method according to claim 4, wherein the tumor cells are transduced with the amplicon *ex vivo*, further comprising the step of introducing the transduced tumor cells into the patient.

6. The method according to claim 4, wherein the amplicons are injected into the site of the tumor cells *in vivo*.

7. The method according to any of claims 1 to 6, wherein the immunomodulatory protein is a cytokine.

8. The method according to claim 7, wherein the cytokine is interleukin-2.

9. The method according to claim 7, wherein the cytokine is granulocyte-macrophage colony stimulating factor.

10. The method according to claim 7, wherein the immunomodulatory protein is a chemokine.

11. The method according to claim 10, wherein the chemokine is RANTES.

12. The method according to any of claims claim 1 to 6, wherein the immunomodulatory protein is a intercellular adhesion molecule.

13. The method according to claim 12, wherein the intracellular adhesion molecule is ICAM-1.

14. The method according to any of claims 1 to 6, wherein the immunomodulatory protein is a costimulatory factor.

15. The method according to claim 14, wherein the costimulatory factor is B7.1.

16. The method according to any of claims1 to 15, wherein a population of tumor cells is transduced with a plurality of species of amplicons containing the genes for the immunomodulatory protein and the additional therapeutic gene.

17. The method according to any of claims 1 to 16, wherein the additional therapeutic gene encodes a second immunomodulatory protein.

18. The method according to any of claims 17, wherein the tumor cells are transduced with amplicons encoding and expressing at least two species of cytokines.

19. The method according to claim 18, wherein tumor cells are transduced with amplicons containing the genes for interleukin-2 and interleukin-12.

20. The method according to claim 18, wherein the tumor cells are transduced with amplicons encoding and expressing a cytokine and a costimulatory factor.

21. The method according to claim 20, wherein tumor cells are transduced with amplicons containing the genes for RANTES and B7.1.

22. The method according to any of claims 1-21, wherein the tumor cells are hepatoma cells or lymphoma cells.

23. A mixture containing a plurality of species of herpes simplex virus amplicons, including at least a first species of amplicon containing the gene for at least one immunomodulatory protein and a second species of amplicon containing the gene for an additional therapeutic gene product.

24. The mixture according to claim 23, wherein the immunomodulatory protein is a cytokine.

25. The mixture according to claim 24, wherein the cytokine is interleukin-2 or granulocyte macrophage colony stimulating factor.

26. The mixture according to claim 23, wherein the immunomodulatory protein is a chemokine.

27. The mixture according to claim 26, wherein the chemokine is RANTES.

28. The mixture according to claim 23, wherein the immunomodulatory protein is a intercellular adhesion molecule.

29. The mixture according to claim 28, wherein the intracellular adhesion molecule is ICAM-1.

30. The mixture according to claim 23, wherein the immunomodulatory protein is a costimulatory factor.

31. The mixture according to claim 30, wherein the costimulatory factor is B7.1.

32. The mixture according to any of claims claim 23 - 31, wherein the additional therapeutic gene encodes a second immunomodulatory protein.

33. The mixture according to any of claims claim 23- 32, wherein the first and second species of amplicons contains genes encoding for RANTES and B7.1.

34. The mixture according to any of claims claim 23- 32, wherein the first and second species of amplicons contains genes encoding for at least two species of cytokines.

35. The mixture according to claim 34, wherein the amplicons contain genes encoding for interleukin-2 and interleukin-12.

36. Tumor cells transduced in accordance with the methods of any of claims 1 to 22.

37. Tumor cells transduced with a mixture of herpes simplex virus amplicons in accordance with any of claims 23 to 35.

38. A method for production of an autologous vaccine to tumor cells comprising transducing the tumor cells with a herpes simplex virus amplicon containing the gene for an immunomodulatory protein to provide transient expression of the immunomodulatory protein by the cells, wherein the immunomodulatory protein is selected from among chemokines, intercellular adhesion molecules and costimulatory factors.

39. The method according to claim 1, wherein the tumor cells are transduced with the herpes simplex amplicons *ex vivo*.

40. The method according to claim 1, wherein the tumor cells are transduced with the herpes simplex cell *in vivo*.

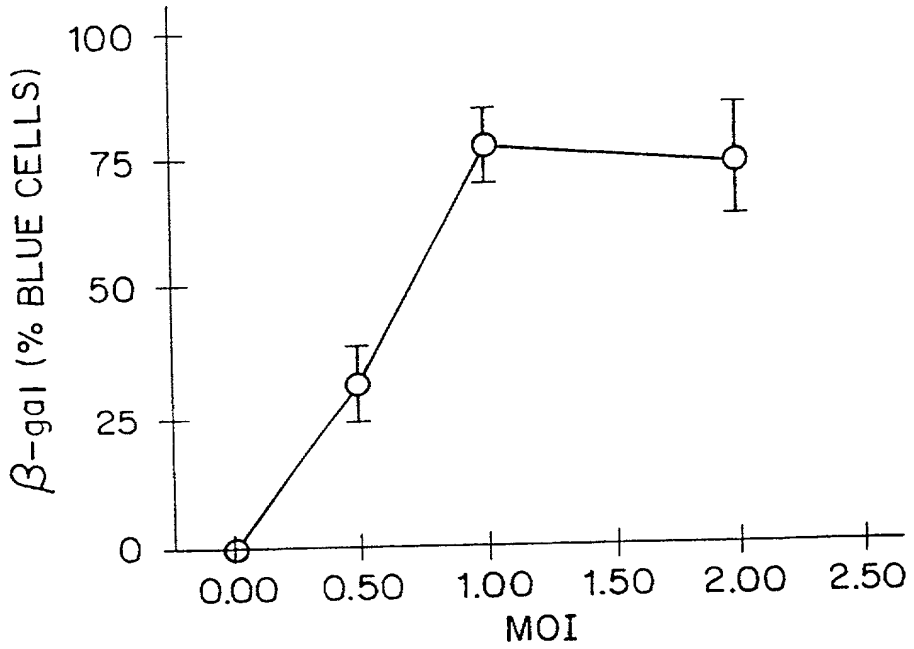
β-gal (% BLUE CELLS)
100
75
50
25
0
0.00
0.50
1.00
1.50
2.00
2.50
MOI

FIG. 1A

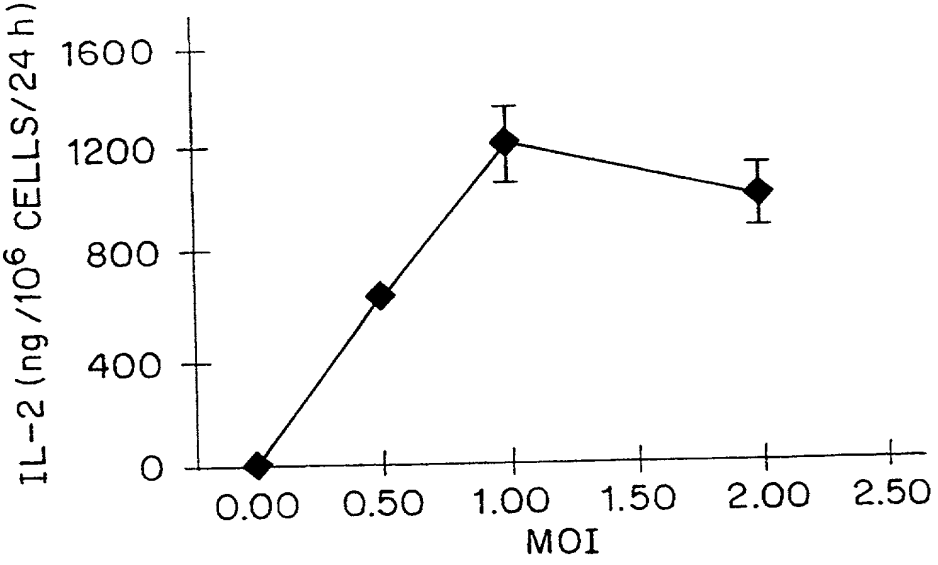
IL-2 (ng /10^6 CELLS/24 h)
1600
1200
800
400
0
0.00
0.50
1.00
1.50
2.00
2.50
MOI

FIG. 1B

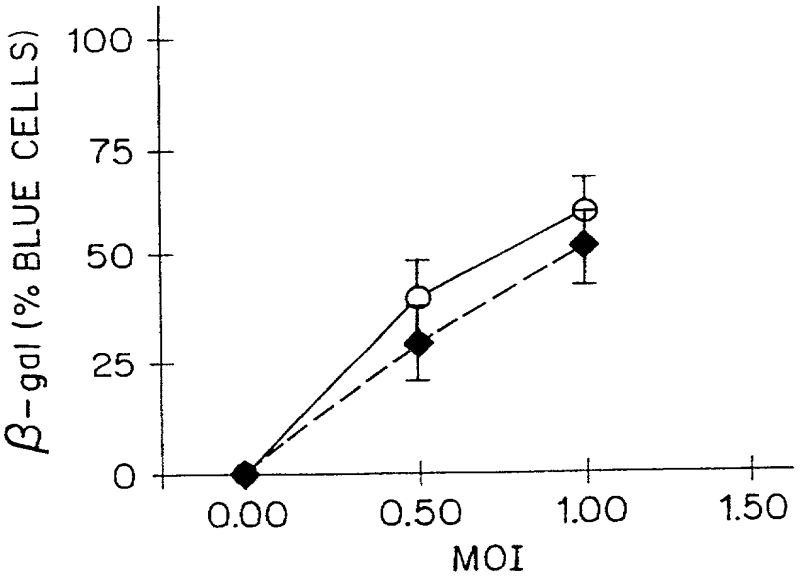

FIG. 1C

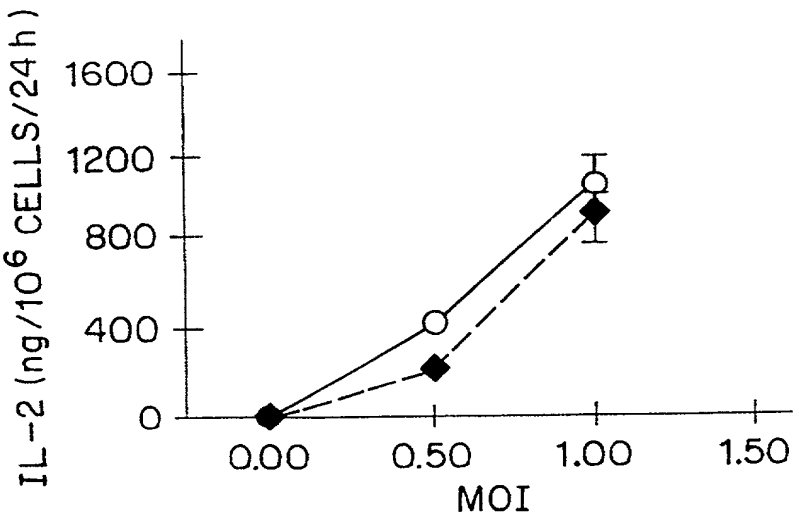

FIG. 1D

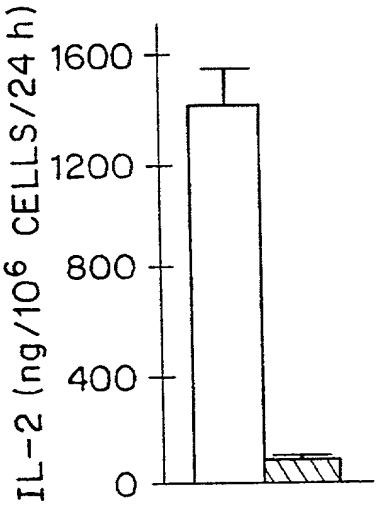

FIG. 1E

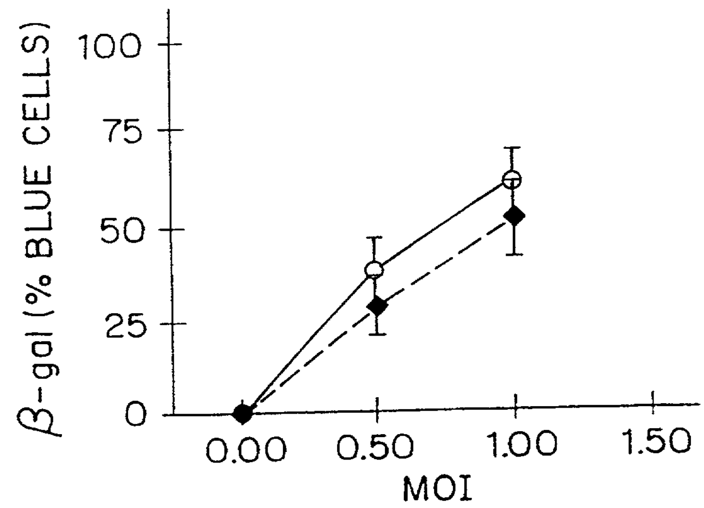

FIG. 2A

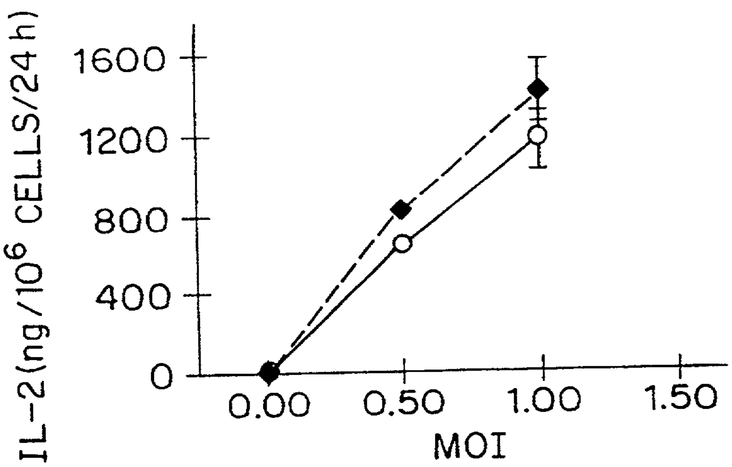

FIG. 2B

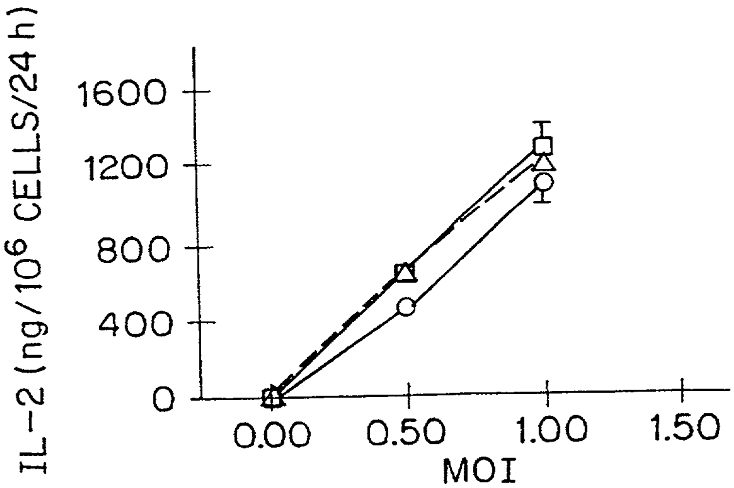

FIG. 2C

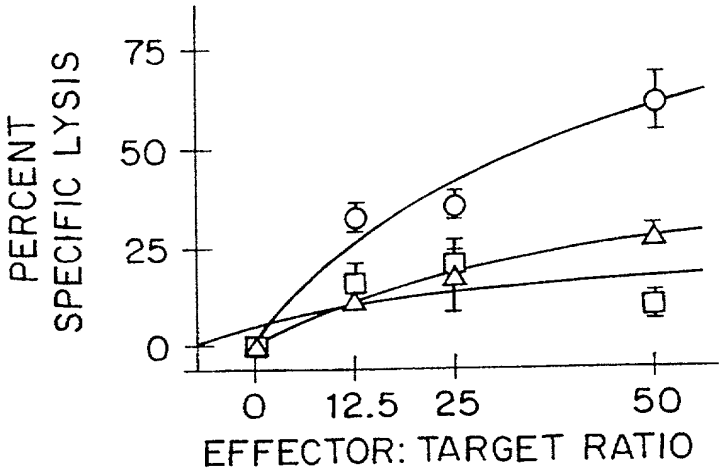

FIG. 3A

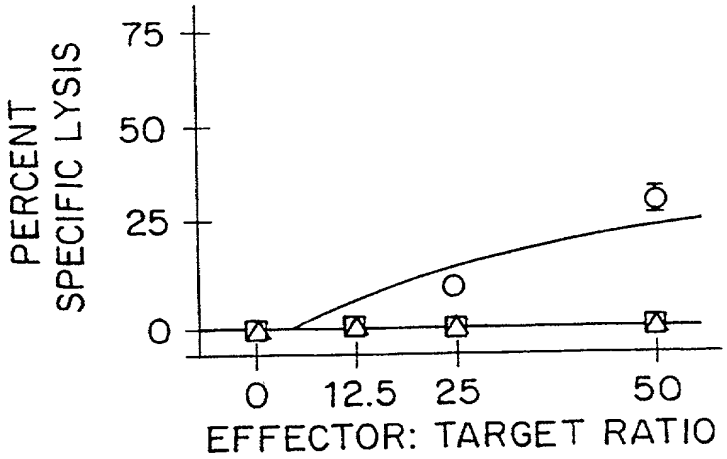

FIG. 3B

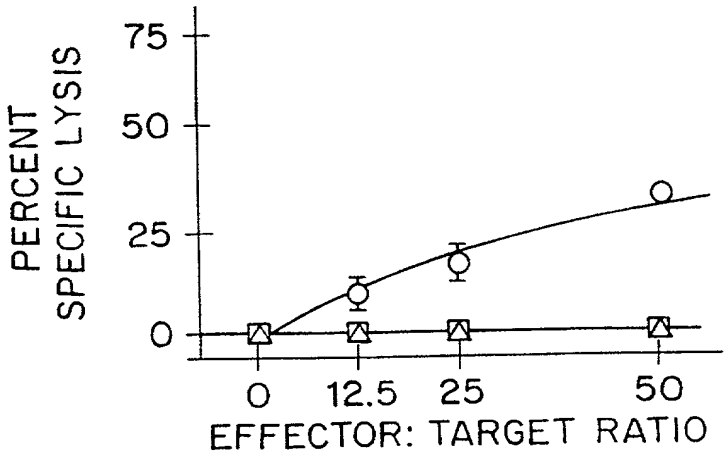

FIG. 3C

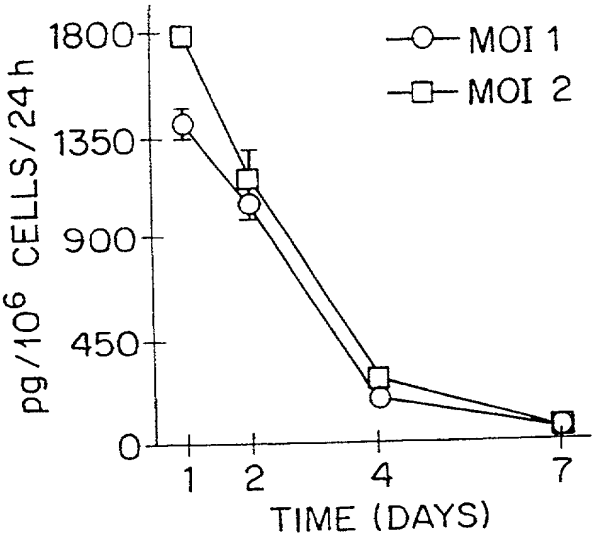

FIG. 4A

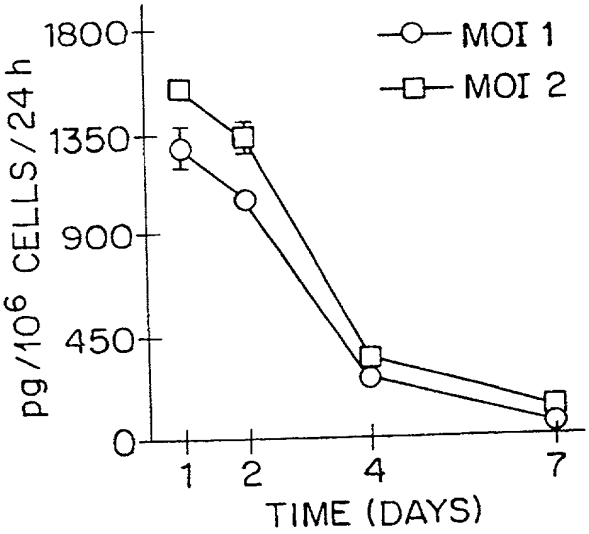

FIG. 4B

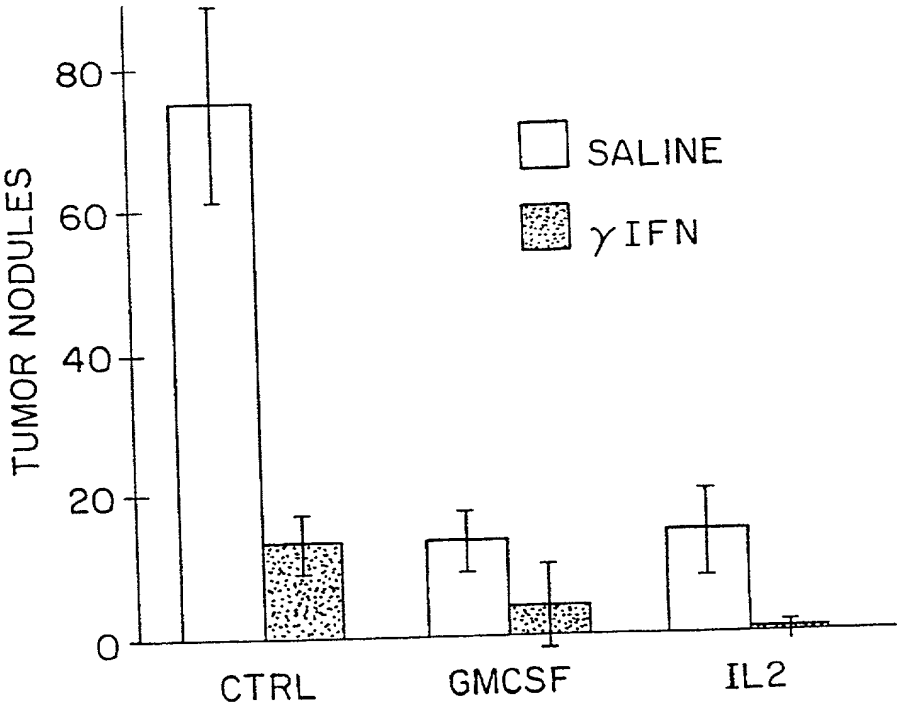

FIG. 5

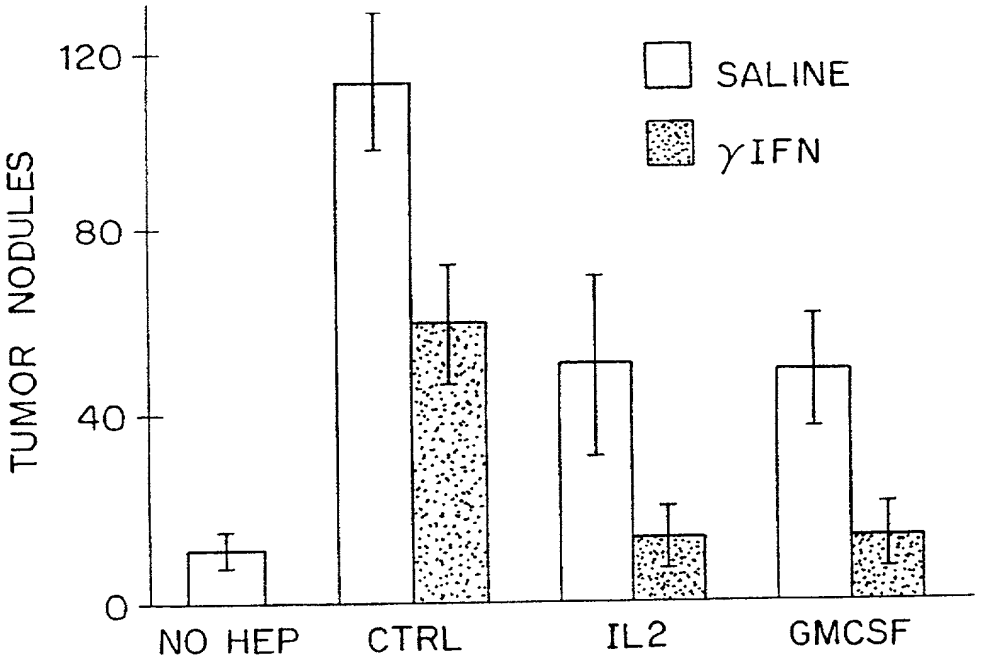
120
80
40
0
TUMOR NODULES
SALINE
γIFN
NO HEP
CTRL
IL2
GMCSF

FIG. 6

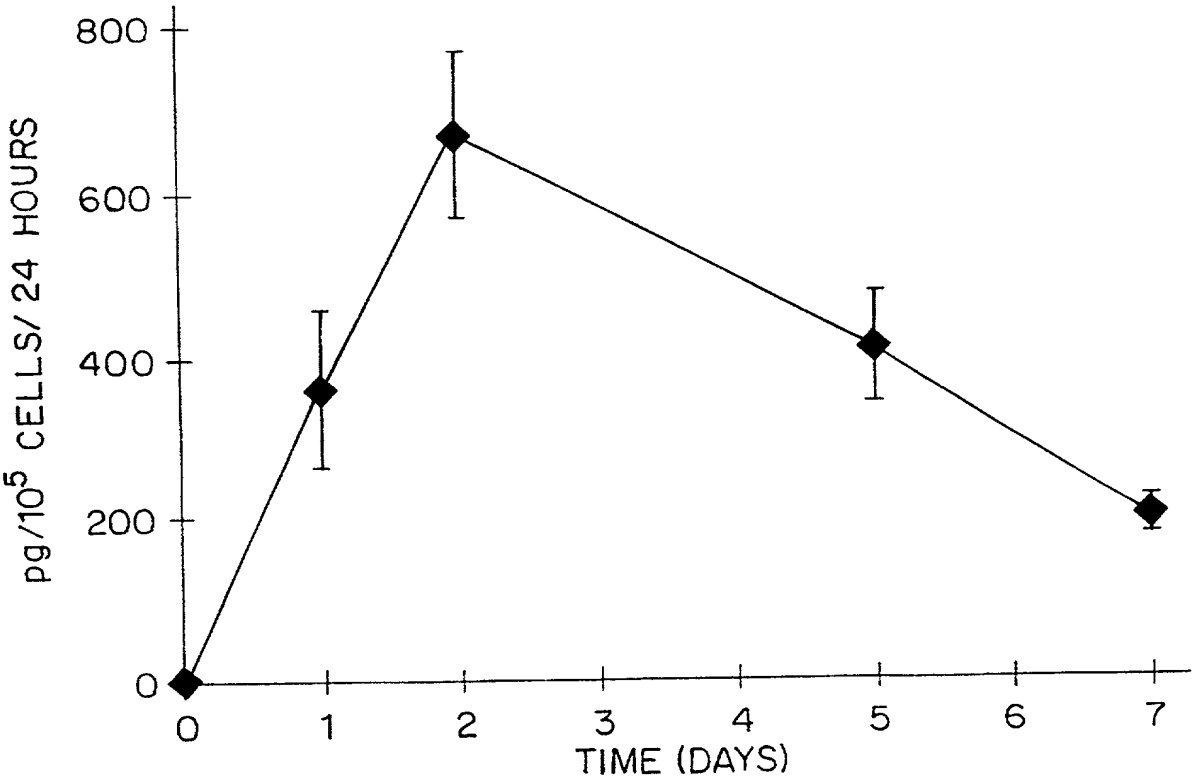
800
600
400
200
0
pg/10^5 CELLS/ 24 HOURS
0
1
2
3
4
5
6
7
TIME (DAYS)

FIG. 7

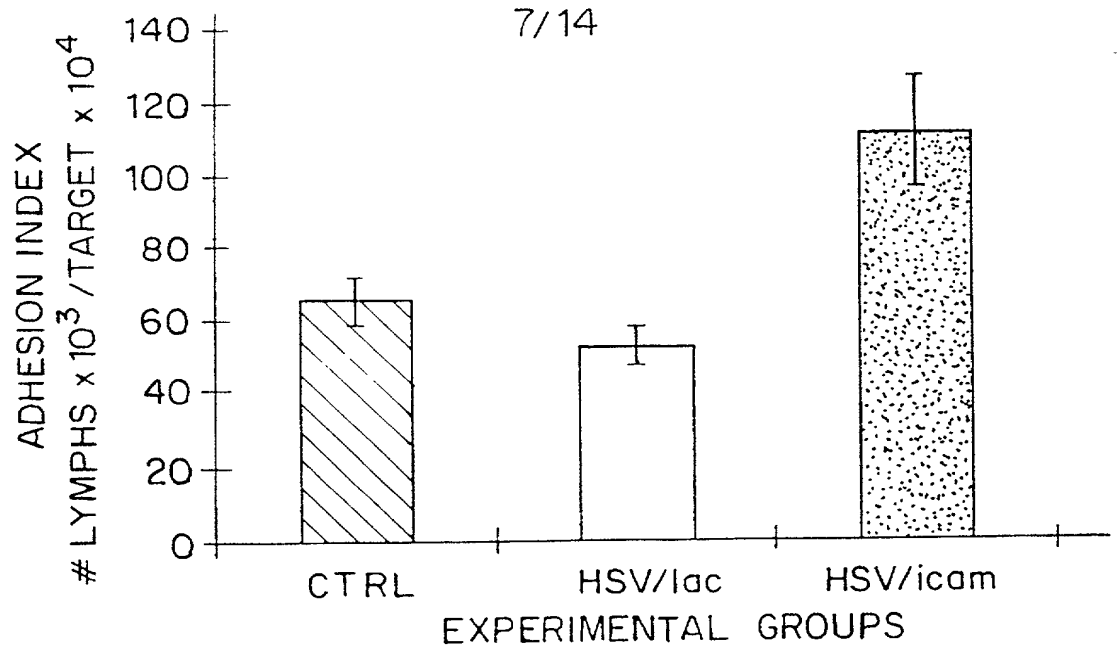
ADHESION INDEX
LYMPHS x 10^3 /TARGET x 10^4
140
120
100
80
60
40
20
0
CTRL
HSV/lac
HSV/icam
EXPERIMENTAL GROUPS

FIG. 8

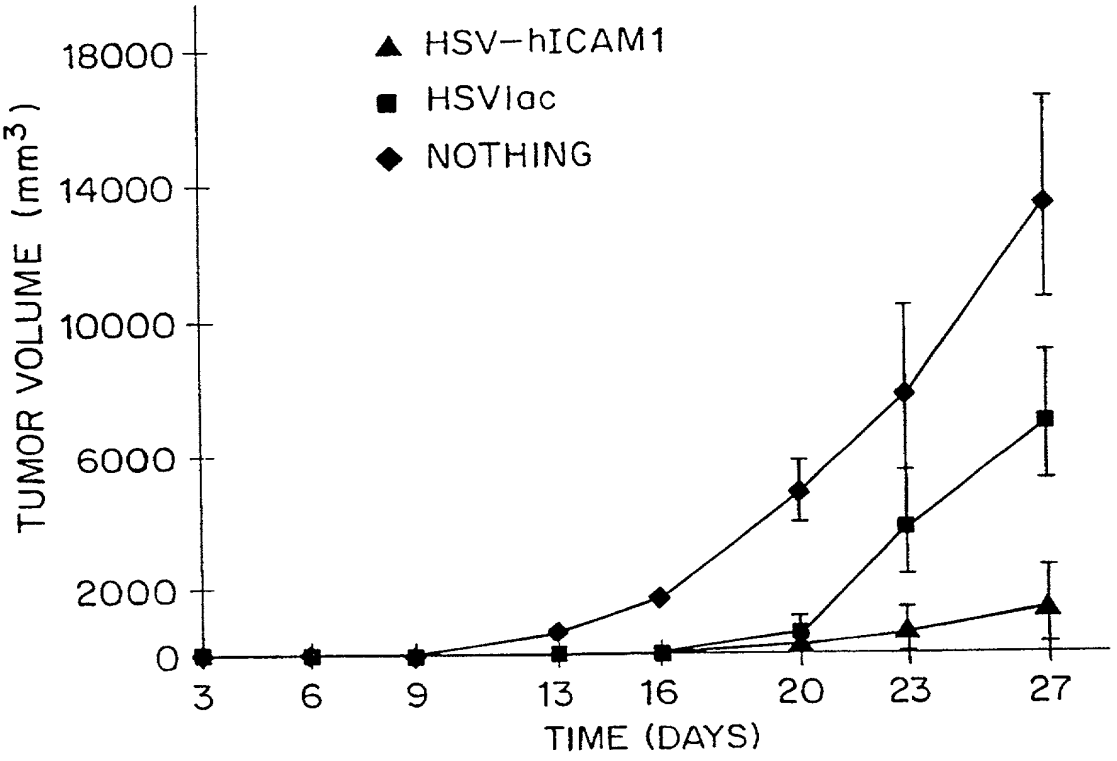
HSV-hICAM1
HSVlac
NOTHING
TUMOR VOLUME (mm^3)
18000
14000
10000
6000
2000
0
3
6
9
13
16
20
23
27
TIME (DAYS)

FIG. 9

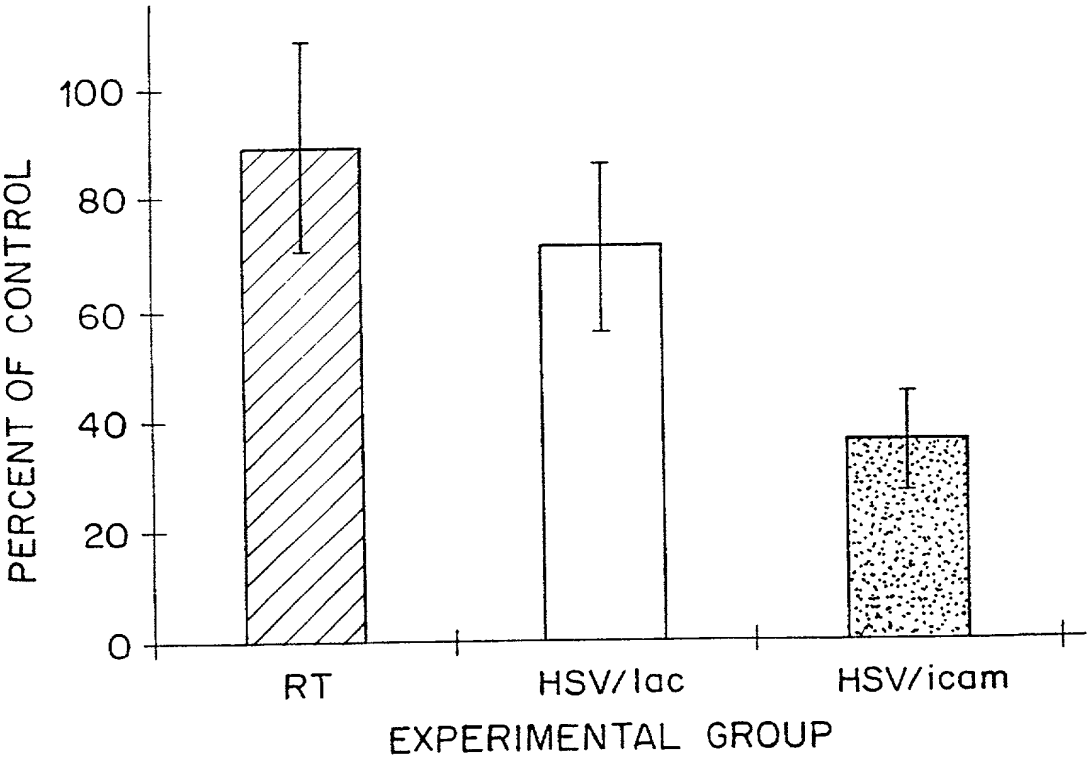

FIG. 10

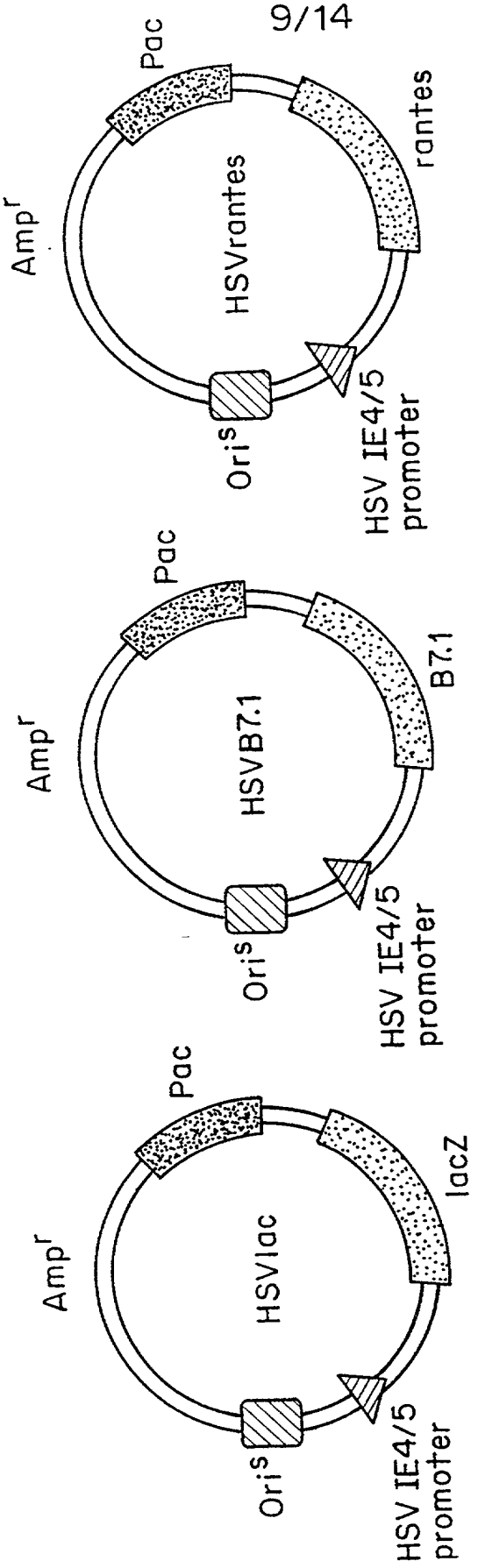
Pac
rantes
HSVrantes
Amp^r
Ori^s
HSV IE4/5
promoter
Pac
B7.1
HSVB7.1
Amp^r
Ori^s
HSV IE4/5
promoter
Pac
lacZ
HSVlac
Amp^r
Ori^s
HSV IE4/5
promoter

FIG. 11

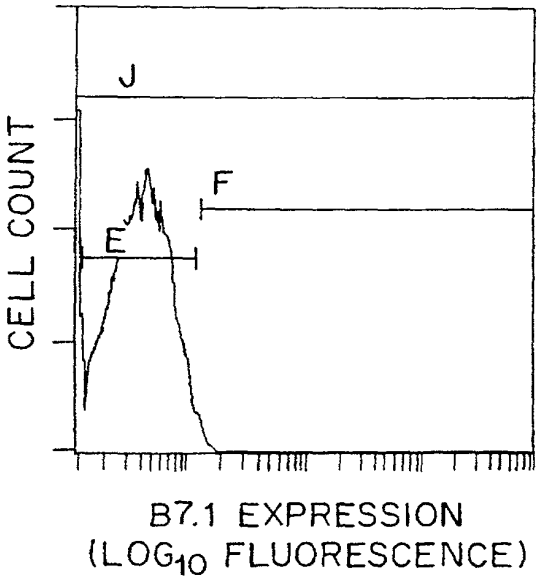

FIG. 12A

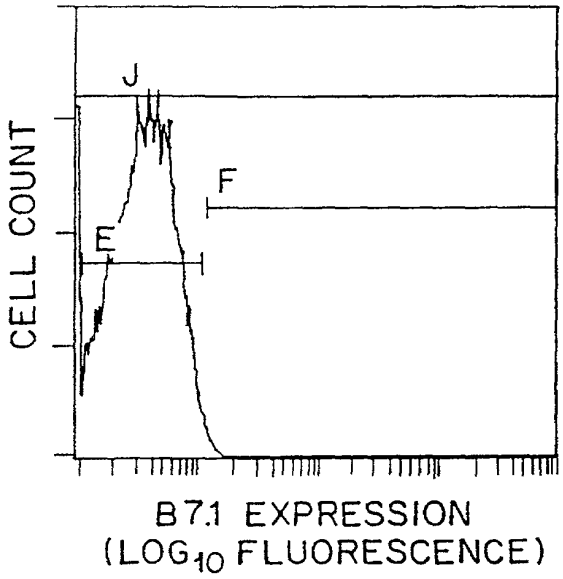

FIG. 12B

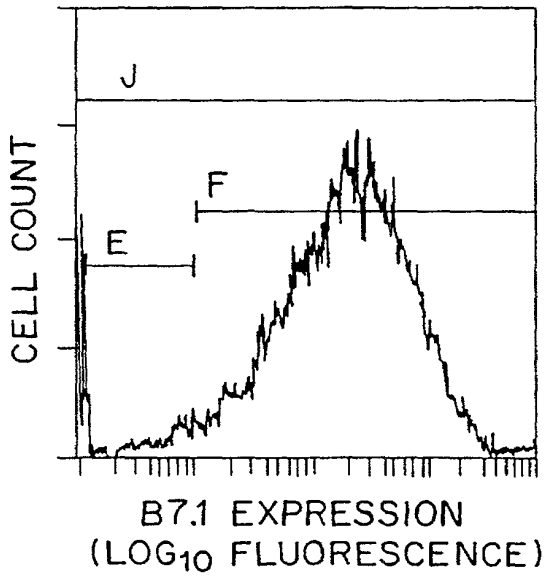

FIG. 12C

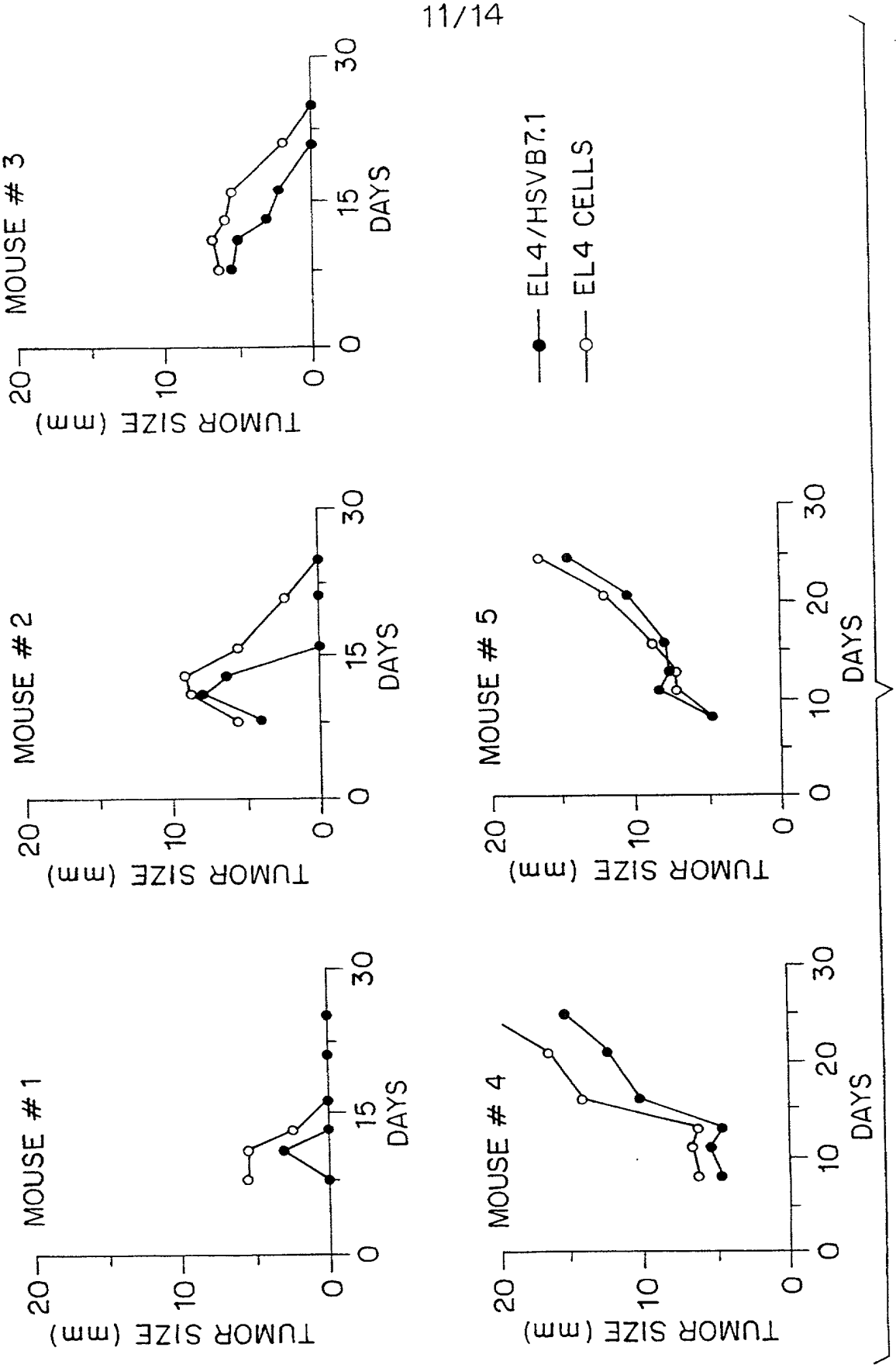
MOUSE # 1
MOUSE # 2
MOUSE # 3
MOUSE # 4
MOUSE # 5
TUMOR SIZE (mm)
DAYS
0
10
20
30
EL4/HSVB7.1
EL4 CELLS

FIG. 13A

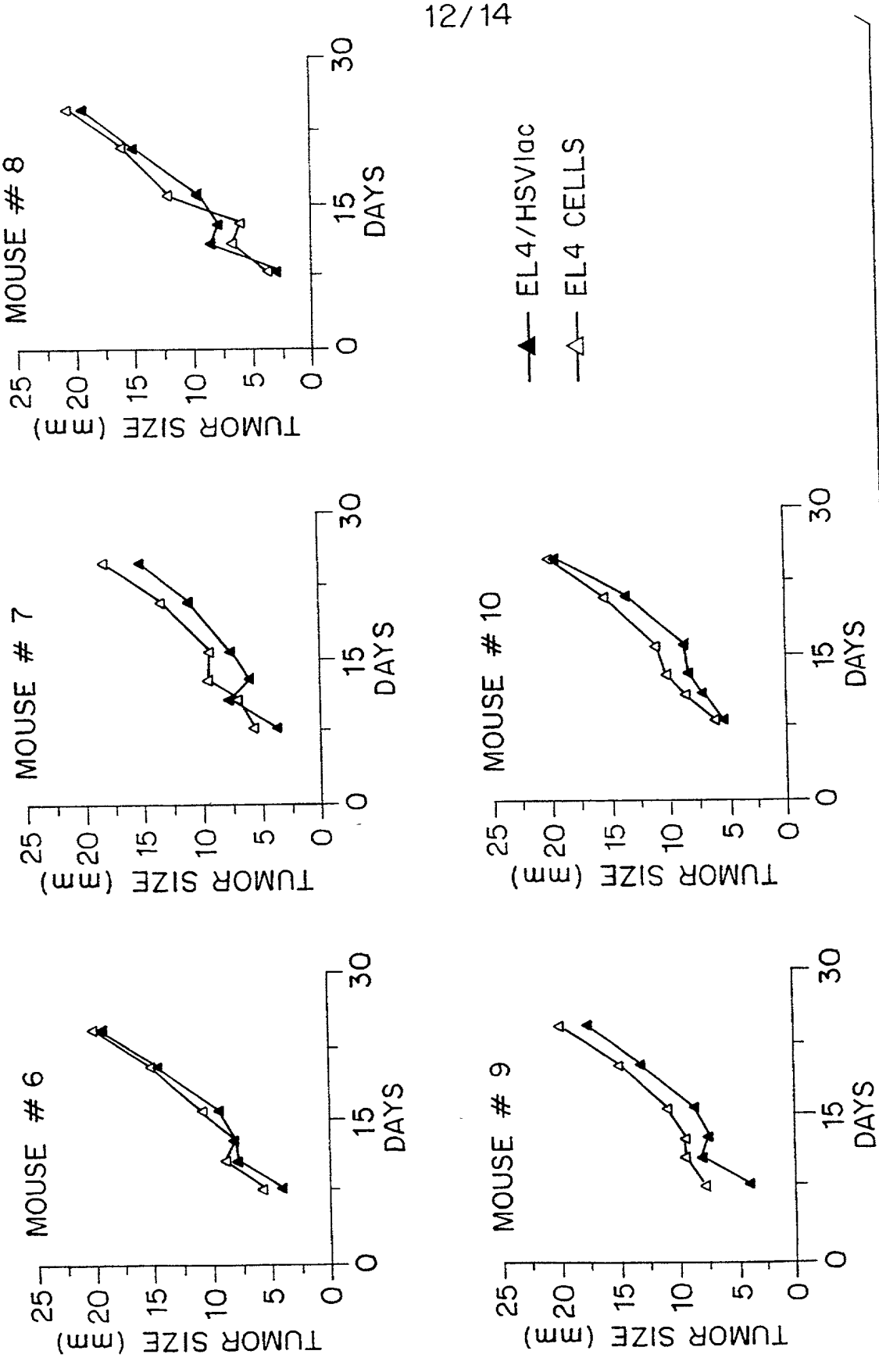
MOUSE # 6
MOUSE # 7
MOUSE # 8
MOUSE # 9
MOUSE # 10
TUMOR SIZE (mm)
DAYS
0
5
10
15
20
25
30
EL4/HSVlac
EL4 CELLS

FIG. 13B

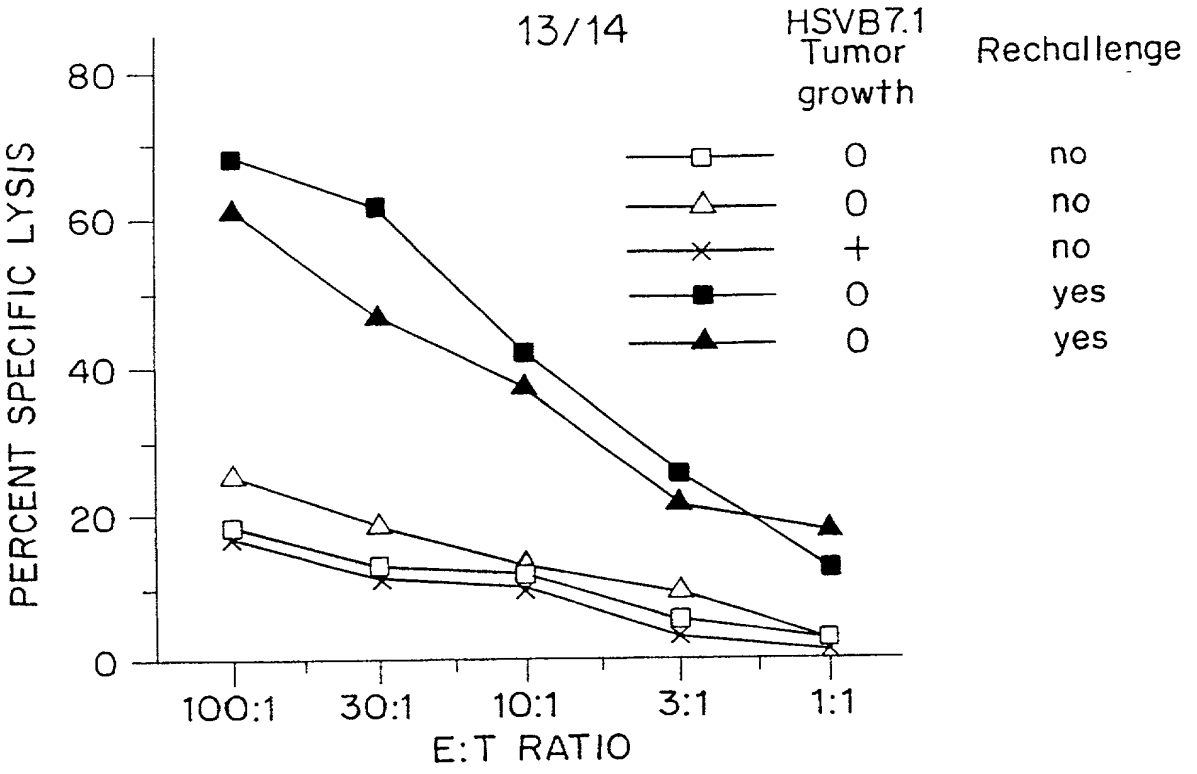

FIG. 14A

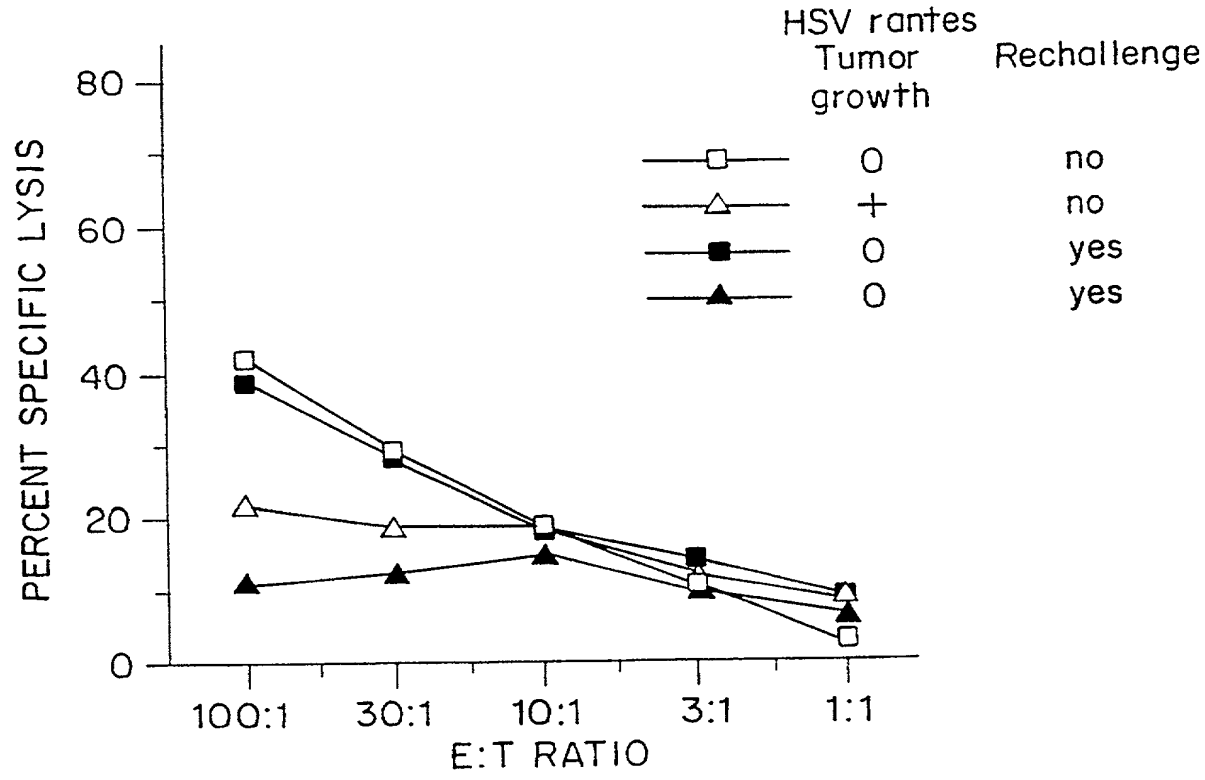

FIG. 14B

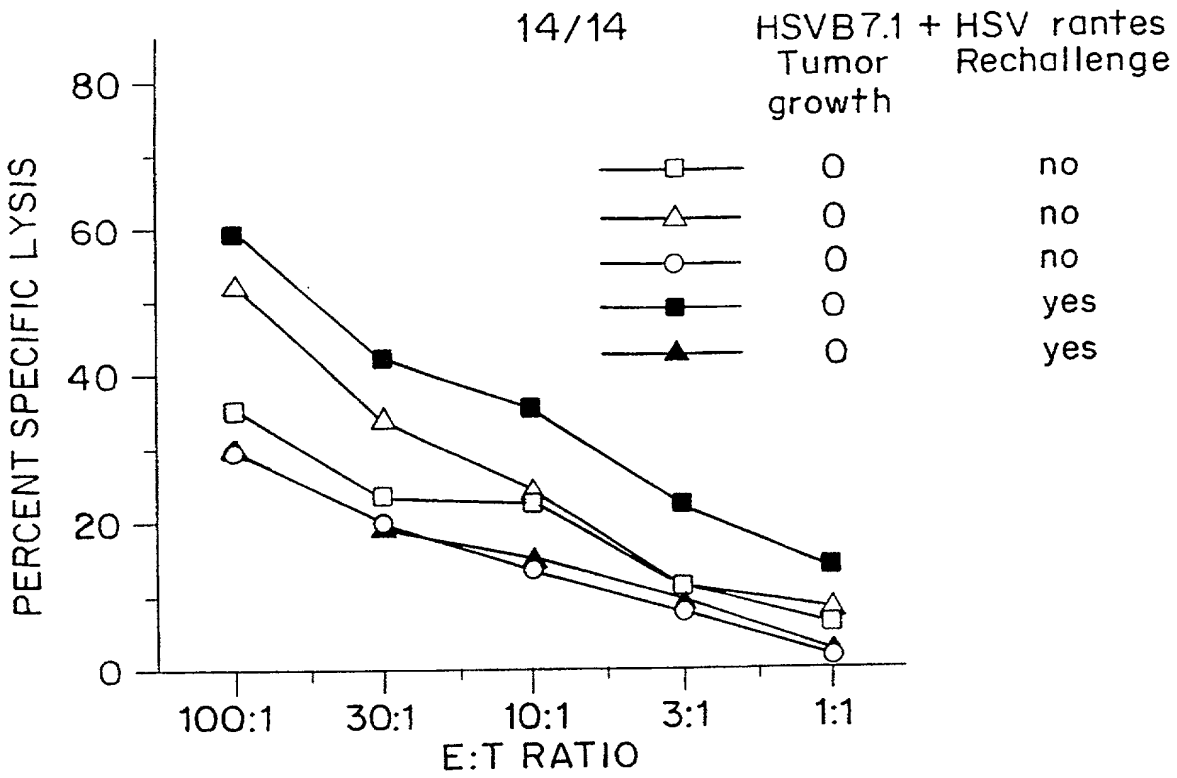

FIG. 14C

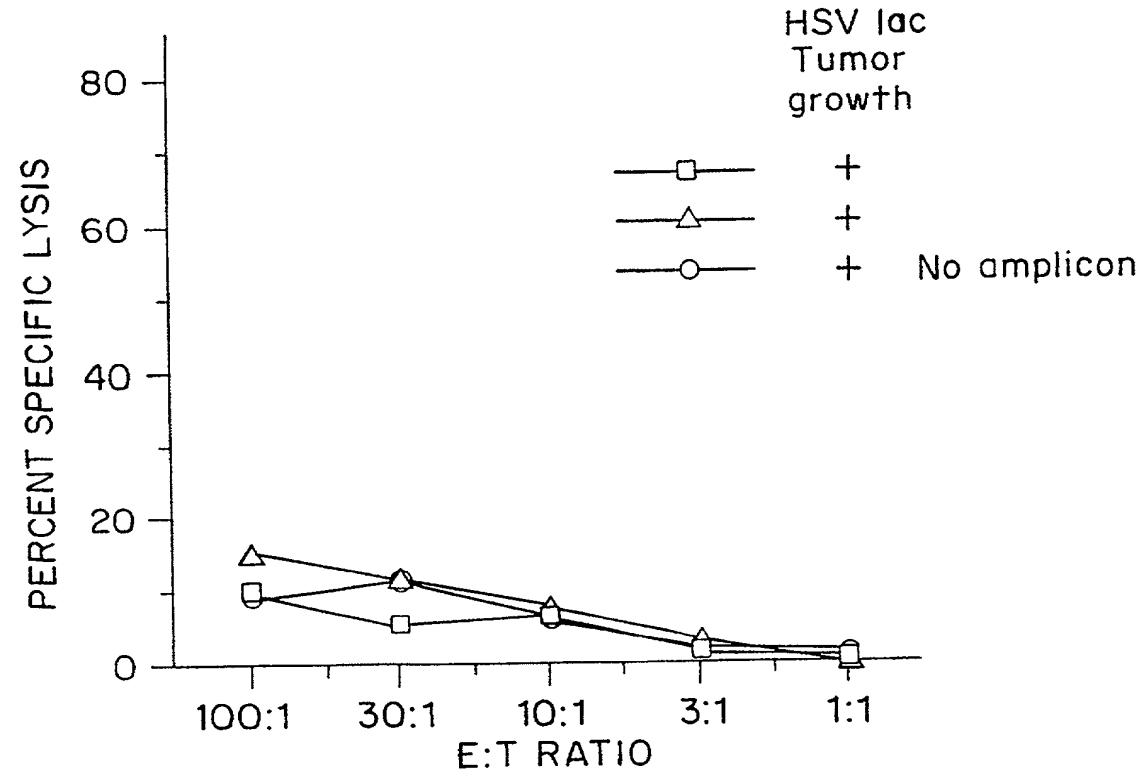

FIG. 14D

OPPEDAHL & LARSON

FILE NO. MSKP031USNP
INVENTOR FONG, ET AL

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My citizenship, residence and post office address are as listed below next to my name.

I believe I am the original, first and [ ] sole/[x] joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: Rapid Production of Autologous Tumor Vaccines by using HSV Amplicon Vectors

the specification of which

(a) [ ] is attached hereto.

(b) [X] was filed on 9/21/99 as Application Serial No. 09/351,556 and was amended on _____.

(c) [X] was described and claimed in International Application No. PCT/US98/05505 filed on March 20, 1998 and amended on October 1, 1998.

Acknowledgment of Duty of Disclosure

I hereby state that I have reviewed and understood the content of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56(a).

35 U.S.C. § 120

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)(patented,pending,abandoned)	(Patent No. if applicable)
(Application Serial No.)	(Filing Date)	(Status)(patented,pending,abandoned)	(Patent No. if applicable)

Power of Attorney

I hereby appoint Carl Oppedahl, PTO Reg. NO. 32,746, Marina T. Larson, PTO Reg. No. 32,038, and Nancy J. Parsons, PTO Reg. No. 40,364 of the firm of OPPEDAHL & LARSON LLP, having office at P.O. Box 5270, 511 Main Street, Frisco, CO 80443 as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
OPPEDAHL & LARSON LLP P.O. Box 5270 Frisco, CO 80443-5270	OPPEDAHL & LARSON LLP (970) 468-2050

OPPEDAHL & LARSON

FILE NO. MSKP031USNP
INVENTOR FONG, ET AL.

Claim for Priority

I hereby claim foreign priority benefits under 35 U.S.C. § 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign applications for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

EARLIEST FOREIGN APPLICATION(S), FILED WITHIN TWELVE MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION					
COUNTRY	APPLICATION NO.	DATE OF FILING (day/month/year)	DATE OF ISSUE (day/month/year)	PRIORITY CLAIMED	CERTIFIED COPY ATTACHED
				YES[ ] NO[ ]	YES[ ] NO[ ]
FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION					
COUNTRY	APPLICATION NO.	DATE OF FILING (day/month/year)	DATE OF ISSUE (day/month/year)		

Provisional Application

I hereby claim the benefit under 35 U.S.C § 119(e) of any United States provisional application(s) listed below.

60/044,005 (application number) March 21, 1997 (filing date)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	FONG	YUMAN	
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE	STATE OR COUNTRY OF RESIDENCE	COUNTRY OF CITIZENSHIP
	NEW YORK NY	US	US
POST OFFICE ADDRESS		CITY	STATE/COUNTRY ZIP CODE
c/o Office of Industrial Affairs Memorial Sloan Kettering Cancer Center 1275 York Avenue		NEW YORK	NY 10021
DATE	11/30/99	SIGNATURE	

[X] Signature for additional joint inventor attached. Number of Pages 1.
[ ] Signature by Administrator(trix) or legal representative for deceased or incapacitated inventor. Number of Pages ___.
[ ] Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR § 1.47. Number of Pages ___.

OPPEDAHL & LARSON

FILE NO. M&KP031USNP
INVENTOR FONG, ET AL

NAME OF SECOND INVENTOR	LAST NAME FEDEROFF	FIRST NAME HOWARD	MIDDLE NAME
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE ROCHESTER NY	STATE OR COUNTRY OF RESIDENCE US	COUNTRY OF CITIZENSHIP US
POST OFFICE ADDRESS 68 Whitewood Lane		CITY ROCHESTER	STATE/COUNTRY ZIP CODE NY 14618
DATE 11/30/99		SIGNATURE	

NAME OF THIRD INVENTOR	LAST NAME ROSENBLATT	FIRST NAME JOSEPH	MIDDLE NAME D.
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE ROCHESTER NY	STATE OR COUNTRY OF RESIDENCE US	COUNTRY OF CITIZENSHIP US
POST OFFICE ADDRESS 88 Southern Parkway		CITY ROCHESTER	STATE/COUNTRY ZIP CODE NY 14618
DATE 12/8/99		SIGNATURE	

NAME OF FOURTH INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE	STATE OR COUNTRY OF RESIDENCE	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		CITY	STATE/COUNTRY ZIP CODE
DATE		SIGNATURE	

NAME OF FIFTH INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE	STATE OR COUNTRY OF RESIDENCE	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		CITY	STATE/COUNTRY ZIP CODE
DATE		SIGNATURE	

OPPEDAHL & LARSON LLP

Applicant or Patentee: Fong, et al. Attorney's Docket No. MSKP031USNP
Serial or Patent No.: 09/381,556 Filed or Issued: September 21, 1999
For: Rapid Production of Autologous Tumor Vaccines by Using HSV Amplicon Vectors

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Sloan-Kettering Institute for Cancer Research
ADDRESS OF ORGANIZATION 1275 York Avenue
New York, NY 10021

TYPE OF ORGANIZATION

( ) UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
(X) TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
( ) NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
( ) WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
( ) WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the above-captioned invention which is described in

( ) the specification filed herewith
(X) Application Serial No. 09/381,556, filed September 21, 1999
( ) Patent No. _____, issued _____

I hereby declare that the rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME James S. Quirk
ADDRESS 1275 York Avenue, New York, NY 10021
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN (X) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)). I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING James S. Quirk

TITLE Senior Vice President, Research Resources Management

ADDRESS OF PERSON SIGNING 1275 York Avenue, New York, NY 10021

SIGNATURE James S. Quirk DATE 10/26/99

OPPEDAHL & LARSON LLP

Applicant or Patentee: Fong , et al. Attorney's Docket No. MSKP031USNP
Serial or Patent No.: _____ Filed or Issued: _____
For: Rapid Production of Autologous Tumor Vaccines by Using HSV Amplicon Vectors

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION University of Rochester
ADDRESS OF ORGANIZATION 518 Hylan Building
Rochester, NY 14627

TYPE OF ORGANIZATION

(X) UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
( ) TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
( ) NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
( ) WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
( ) WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the above-captioned invention which is described in

( ) the specification filed herewith
(X) Application Serial No. _____, filed _____
( ) Patent No. _____, issued _____

I hereby declare that the rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention. If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)). I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Mark Coburn
TITLE Interum Director, Office of Technology Transfer
ADDRESS OF PERSON SIGNING 518 Hylan Builkding, Rochester, NY 14627
SIGNATURE Mark S. Coburn DATE 10-4-99

United States Patent & Trademark Office

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